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13. ABSTRACT (Maximum 200 Words)

This final progress report covers the activities of three projects and one core resource. We provide the required reporting elements for each of these individuals. The following summarizes the key accomplishments from this award (DAMD17-00-1-0102). We demonstrated in Project A that somatic mutations of the SRD5A2 gene are fairly common; some are recurrent and based on *in vitro* functional studies, dramatically change enzymatic kinetics. We demonstrated in Project B that somatic mutations in the AR gene are extremely rare across all racial-ethnic groups prior to hormonal ablation therapy. We then proceeded to contribute substantially to understanding the molecular biology of AR signaling in the prostate through a series of publications. In Project C we completed immunohistochemical analyses on a series of molecular markers (p21, COX-2, caveolin-1, GRP78) of prostate cancer progression on a population-based multiracial-ethnic sample of patients – statistical analysis are ongoing. In our Epidemiology Core, we collected and processed 522 prostate cancer tissue samples from a multiethnic cohort. This tissue bank (to our knowledge the largest of its kind in a multiethnic context) will be an extremely valuable resource for additional planned analyses.

14. SUBJECT TERMS

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OVERALL INTRODUCTION

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- We demonstrated in Project B that somatic mutations in the AR gene are extremely rare across all racial-ethnic groups prior to hormonal ablation therapy. We then proceeded to contribute substantially to understanding the molecular biology of AR signaling in the prostate through a series of publications.

- In Project C we completed immunohistochemical analyses on a series of molecular markers (p21, COX-2, caveolin-1, GRP78) of prostate cancer progression on a population-based multiracial-ethnic sample of patients – statistical analysis are ongoing.

- In our Epidemiology Core, we collected and processed 522 prostate cancer tissue samples from a multiethnic cohort. This tissue bank (to our knowledge the largest of its kind in a multiethnic context) will be an extremely valuable resource for additional planned analyses.

PERSONNEL PAID ON THIS GRANT

Ross, Ron	USC	PI
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Reitze, Page	University of Hawaii	

REPORTABLE OUTCOMES

The following is a list of publications/manuscripts resulting from this work.

Balk, S. (2002). "Androgen receptor as a target in androgen-independent prostate cancer." Urology **60**(3 Suppl 1): 132-138.

Buchanan, G., N. M. Greenberg, et al. (2001). "Collocation of androgen receptor gene mutations in prostate cancer." Clin Cancer Res **7**(5): 1273-81.

Buchanan, G., R. A. Irvine, et al. (2001). "Contribution of the androgen receptor to prostate cancer predisposition and progression." Cancer Metastasis Rev **20**(3-4): 207-23.

Chen, C. D., D. S. Welsbie, et al. (2004). "Molecular determinants of resistance to antiandrogen therapy." Nat Med **10**(1): 33-39.

Feldman, B. J. and D. Feldman (2001). "The development of androgen-independent prostate cancer." Nat Rev Cancer **1**(1): 34-45.

Jia, L., C. S. Choong, et al. (2004). "Androgen receptor signaling: mechanism of interleukin-6 inhibition." Cancer Res **64**(7): 2619-26.

Jia, L., J. Kim, et al. (2003). "Androgen receptor activity at the prostate specific antigen locus: steroidal and non-steroidal mechanisms." Mol Cancer Res **1**(5): 385-92.

Kim, J., L. Jia, et al. (2003). "Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor." Nucleic Acids Res **31**(23): 6741-7.

Makridakis, NM and Reichardt, JKV (2004) Molecular Epidemiology of Androgen Metabolic Loci in Prostate Cancer and Progression, *J. Urol.* **171**, S25-S29 also benefited from the generous support of this grant.

Makidrakis, NM., Akalu, A., and Reichardt, JKV (2004) Identification and Characterization of Somatic Steroid 5a-Reductase (SRD5A2) Mutations in Human Prostate Cancer Tissue *(provisionally accepted – in revision)*

Park, J. J., R. A. Irvine, et al. (2000). "Breast cancer susceptibility gene 1 (BRCAI) is a coactivator of the androgen receptor." Cancer Res **60**(21): 5946-9.

Xue, W. M., G. A. Coetzee, et al. (2001). "Genetic determinants of serum prostate-specific antigen levels in healthy men from a multiethnic cohort." Cancer Epidemiol Biomarkers Prev **10**(6): 575-9.

Project A. The Human SRD5A2 Gene and Prostate Cancer Progression
PI: Juergen Reichardt, Ph.D.

Introduction

There is a large variation in prostate cancer rates between racial-ethnic groups in the US. We have taken a "candidate gene" approach to prostate cancer. We have focused on androgen-metabolic genes since they can regulate prostatic growth. Specifically, we proposed to examine the hypothesis that *de novo* DNA sequence variations (i.e., somatic mutations) in the type II (or prostatic) steroid 5□-reductase (SRD5A2) gene contribute substantially to the progression of prostate cancer particularly across racial/ethnic lines.

Body

In our application we had proposed to investigate the following three interrelated specific aims:

1. To identify somatic mutations in prostatic tumors of men from four racial/ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos] in the regulatory elements of the SRD5A2 gene, specifically its promoter and the 5' and 3' untranslated regions (UTR);

2. To determine the frequency of somatic SRD5A2 mutations in prostate cancers in four racial-ethnic groups [African-Americans, Asian-Americans, Caucasians and Latinos];

3. To determine the contribution of the SRD5A2 somatic mutations screened for in specific aim 2 to prostate cancer grade and stage of disease as surrogates for outcome.

Key Research Accomplishments

Substantial progress was made toward specific aim 1 as reported over the years of this grant. Highlights include the identification and characterization of 10 *de novo* somatic mutations (cf. Fig. 1).

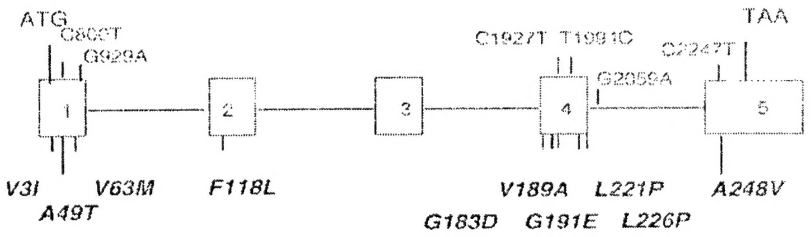

Figure 1: *De novo* somatic mutations identified in the SRD5A2 gene. The ten amino acid substitutions characterized in Table 1 are shown below the gene in the single letter amino acid code and bold lettering.

Furthermore, we have now reconstructed all *de novo* somatic amino acid substitutions in the SRD5A2 cDNA. Specifically, we have reconstructed by site-directed mutagenesis 10 single amino acid substitutions and verified them by site-directed mutagenesis. We have also overexpressed and characterized them (Table 1).

Table 1
In Vitro Characterization of SRD5A2 Somatic Mutations

Variant	V_{max} (nmol/min/mg)	K_m; T (micromolar)	K_m; NADPH (micromolar)	pH optimum
WT	2 (1.7-2.2)	0.6 (0.5-0.8)	8 (6-14)	6.0
V3I	5	3	12	5.5-6.0
A49T	9.8	1.5	7	6.0
V63M	1.6	1.5	11	6.0
F118L	8.2	4.5	17	6.0-6.5
G183D	1.8	1.4	19	6.0
V189A	0.8	1.3	39	6.0-6.5
G191E	0.9	1	25	6.0
L221P	0.7	0.7	31	6.5
L226P	1.8	0.3	22	6.0
A248V	6.3	1.4	5	6.0

Note: WT is the normal (wild type) enzyme and the range of values is shown in parentheses.

The extensive characterization indicates a 14-fold variation in the enzyme activity expressed as the apparent V_{max}, ranging from 9.8 nmol/mimn/mg (for the A49T mutation) to 0.7 nmol/mimn/mg (L221P; Table 1). Significant variations of the apparent K_m for the substrate (testosterone; T) and cofactor (NADPH; Table 1) were also identified.

We also made significant progress toward specific aims 2 and 3: we have genotyped 186 DNA tissue samples across racial ethnic groups and identified 14 mutations, half in normal and half in paired tumor tissue. Four pairs contained mutations and both normal and tumor tissue A49T was a recurring mutation found in 3 samples.

Reportable Outcomes

1. A manuscript detailing the somatic mutations (cf. Fig. 1 and Table 1) has been submitted. It is currently in revision at the journal.
2. A review by Makridakis, NM and **Reichardt**, JKV (2004) Molecular Epidemiology of Androgen Metabolic Loci in Prostate Cancer and Progression, *J. Urol.* **171**, S25-S29 also benefited from the generous support of this grant.

Conclusions

This laboratory has completed specific aim 1. We also characterized the biochemical properties of the somatic mutations we identified in the SRD5A2 locus. In specific aims 2 and, we have screened for the A49T and V63M recurrent protein-coding mutations in tumor and "normal" samples obtained through this grant. Both were found repeatedly as outlined in Fig. 1.

Appendices

The published paper and in press paper are attached.

Project B. Androgen Receptor (AR) Signaling in Prostate Cancer Progression
PI: Gerhard Coetzee, Ph.D.

Introduction

The androgen receptor (AR) is the single most important determinant of androgen ablation failure in prostate cancer since it provides mechanisms of escape from the ablation. The role of the AR as a determinant of antiandrogen therapy resistance was recently dramatically demonstrated when increased expression of the AR was the only consistent change observed in microarray-based profiles of resistant PCa xenograft models – increased AR mRNA and protein was both necessary and sufficient to convert PCa to a hormone-refractory stage (Chen *et al.*, 2004). In contrast to breast cancer, where both estrogen receptor-positive and -negative tumors are found, prostate tumors are almost always AR positive, even during so-called androgen-independent phases of the disease. Resistance to androgen ablation therapies is not due to a loss of androgen sensitivity but rather results from mutation or amplification of the AR gene, altered interaction of coregulatory molecules with the AR, and/or non-steroidal activation of the AR which consequently lead to a deregulated androgen signaling axis [reviewed in (Buchanan *et al.*, 2001; Feldman and Feldman, 2001; Balk, 2002)]. Although non-steroidal activation of the AR might account for as much as 50% of all prostate cancers that fail ablation therapy, the mechanisms involved are poorly understood. It is thought (although little mechanistic detail is available) that signaling via growth factors (such as EGF) and cytokines (such as IL-6) modulate the AR in a non-steroidal manner leading to AR-mediated gene expression at low, castrate levels of androgen.

Body

AR mutations

Our original plan was to sequence the AR in 480 prostate cancer tumor blocks. To date we have sequenced 90 specimens for mutation in 6 hotspot areas previously identified in an analysis of the literature (Buchanan *et al.*, 2001). The 6 hotspot areas encompass aa N36-Q96; Q260-P279; P479-M537; L659-G683; D690-V736; I686-Thr918. Although a few 'mutations' were detected during the first screen, none of them could be verified by repeat PCR preparation of the same sample. Therefore we have terminated the sequencing part of the project. Our conclusion is that if AR mutations do in fact exist in prostate tumors, they are either in the non-hotspot areas or are at such a low frequency that not much can be concluded by their presence. More recently it is thought that AR mutations are generally selected for during and by androgen ablation treatment that imposes a strong selection pressure on the tumor, allowing outgrowth of clones harboring AR mutations. Since none of our tumor material was obtained from men who have failed androgen ablation treatment, this question could not be addressed using the present set of samples. As a consequence we have concentrated on a molecular biology approach in an attempt to understand AR function in the context of

prostate cancer progression. We have made outstanding progress on several fronts. Twelve publications arose from this work and each is summarized below.

1) Park JJ, Irvine, RA, Buchanan G, Koh SS, Park JM, Tilley WD, Stallcup MR, Press MF, **Coetzee GA**. BRCA1 is a coactivator of the androgen receptor. *Cancer Res* 60:5946-5949, 2000.

In this study, the role of BRCA1 in ligand-dependent androgen receptor (AR) signaling was assessed. In transfected prostate and breast cancer cell lines, BRCA1 enhanced AR-dependent transactivation of a probasin-derived reporter gene. The effects of BRCA1 were mediated through the N-terminal activation function (AF-1) of the receptor. Cotransfection of p160 coactivators markedly potentiated BRCA1-mediated enhancement of AR signaling. In addition, BRCA1 was shown to interact physically with both the AR and the p160 coactivator, GRIP1. These findings suggest that BRCA1 may directly modulate AR signaling, and therefore, may have implications regarding the proliferation of normal and malignant androgen-regulated tissues.

2) Xue W, **Coetzee GA**, Ross RK, Irvine RA, Kolonel L, Henderson BE, Ingles SA. Genetic determinants of serum PSA levels in healthy men from a multiethnic cohort. *Cancer Epidemiol Biomarkers Prev* 10:575-579, 2001.

We had previously reported an association between prostate cancer risk and polymorphisms in the prostate-specific antigen (PSA) and androgen receptor (AR) genes. The purpose of this study was to test whether these two polymorphisms, AR CAG, and PSA ARE1, influence serum PSA levels in healthy men. Serum PSA and the two genotypes were assayed for 420 healthy men from a multi-ethnic cohort, and regression models were fit to estimate the effects of AR CAG genotype and PSA ARE1 genotype on serum PSA levels. Predicted serum PSA decreased 3.5% with each additional AR CAG repeat decile ($p=0.01$). Serum PSA was also associated with PSA ARE1 genotype, with PSA levels being higher among men with the PSA AA genotype compared to men with the AG or GG genotypes ($p=0.02$). The relationship between serum PSA level and AR CAG length differed according to PSA genotype ($p=0.049$): for genotype GG, the slope was not significantly different from zero ($p=0.74$); for genotype AG, serum PSA increased 4.5% with each decrease of one CAG repeat decile ($p=0.03$); for genotype AA serum PSA increased 7% with each decrease of one CAG repeat decile ($p=0.02$). These results indicate that in healthy men, genetic variants in the PSA and AR genes contribute to variation in serum PSA levels. Men with the PSA AA genotype and short AR CAG alleles have, on average, higher serum PSA levels.

3) Jia L, Kim J, Shen H, Clark PE, Tilley, WD, **Coetzee, GA**. Androgen receptor activity at the prostate specific antigen locus: Steroidal and non-steroidal mechanisms. *Mol Cancer Res* 1:385-392, 2003.

Ligand-activated androgen receptors (ARs) occupy target genes and recruit histone modifiers that influence transcriptional competency. In LNCaP prostate cancer cells, the natural ligand 5α-dihydrotestosterone (DHT) activates transiently-transfected

AR-responsive promoter constructs; concurrent treatment with the protein kinase A activator forskolin enhanced AR stimulation induced by DHT. Additional treatment with the cytokine IL-6, purportedly an AR activator, markedly inhibited receptor activity. To assess AR activity on natural chromatin-integrated promoters/enhancers, we determined AR occupancy of the endogenous prostate specific antigen (PSA) promoter/enhancer as well as PSA expression in LNCaP cells treated with DHT; AR occupancy of the PSA enhancer was rapid (within 1 hour of stimulation), robust (10-fold over background), and sustained (8-16 hours). In contrast, AR occupancy of the PSA promoter was only increased by 2-fold. Histone H3 acetylation at both the enhancer and promoter was evident 1-2 hours after DHT treatment. Detectable pre- and mature PSA mRNA levels appeared after 1 and 6 hours treatment, respectively. Substantial qualitative and quantitative differences in PSA expression and AR occupancy of the PSA enhancer were observed when DHT-induced and ligand-independent activation of the AR were compared; forskolin stimulated PSA mRNA and protein expression, whereas IL-6 inhibited both DHT- and forskolin stimulated expression. IL-6 did not diminish DHT-dependent AR occupancy of the PSA enhancer but inhibited CBP/p300 recruitment, histone H3 acetylation and cell proliferation. These findings provide a contextual framework for interpreting the contribution of non-steroidal activation of the AR to signaling *in vivo*, and have implications for prostate cancer cell growth.

4) Kim J, Jia L, Tilley WD, **Coetzee GA**. Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor. *Nucleic Acids Res* 31:6741-6747, 2003.

The methylation of histone H3 correlates with either gene expression or silencing depending on the residues modified. Methylated lysine 4 (H3-K4) is associated with transcription at active gene loci. Furthermore, it was reported that trimethylated but not dimethylated H3-K4 is exclusively associated with active chromatin in Saccharomyces cerevisiae. In the present study, we investigated the H3-K4 methylation at the human prostate specific antigen (PSA) locus following gene activation and repression via androgen receptor (AR). We show that ligand-induced, AR-mediated transcription was accompanied by rapid decreases in di- and trimethylated H3-K4 at the PSA enhancer and promoter. Moreover, the observed decreases in H3-K4 methylation were reversed when AR was inhibited by a specific AR antagonist, bicalutamide. In contrast to the decreases in methylation at the 5' transcriptional control regions of the PSA gene, H3-K4 methylation in the coding region steadily increased after a lag period of about 4 hours. The results suggest a novel role of methylated H3-K4 in transcriptional regulation.

5) Jia L, Choong S-Y, Ricciardelli C, Kim J, Tilley WD, **Coetzee GA**. Androgen receptor signaling: mechanism of IL-6 inhibition. *Cancer Res* 64:2619-2626, 2004.

Non-steroidal signaling via the androgen receptor (AR) plays an important role in hormone-refractory prostate cancer. Previously we have reported that the pleiotropic cytokine, IL-6, inhibited DHT-mediated expression of prostate specific antigen (PSA) in

LNCaP cells (Jia *et al.*, *Mol Cancer Res* 1:385-392, 2003, see above). In the present study, we explored the mechanisms involved in this inhibition and considered possible effects on AR nuclear translocation, recruitment of transcription cofactors, and the signaling pathways that may mediate this inhibitory effect. IL-6 neither induced nuclear localization of the AR nor inhibited DHT-induced nuclear translocation of the receptor. IL-6 did not affect AR or p160 coactivator recruitment to the transcription initiation complex on the PSA enhancer and promoter. Moreover, it did not lead to the recruitment of the corepressor SMRT or HDAC1 at the same sites. IL-6 did, however, prevent the recruitment of the secondary coactivator, p300, to the complex and partially inhibited histone H3 acetylation at the same loci. Furthermore, inhibition by IL-6 was not mediated by the MAPK or the Akt pathways and was partially abrogated by STAT3 knock-down using siRNA. Our results show that IL-6 modulates androgen action through the differential recruitment of cofactors to target genes. These findings may account for the pleiotropic actions of IL-6 in malignant prostate cells.

6) Buchanan G, Craft PS, Yang M, Cheong A, Prescott J, Jia L, **Coetzee GA**, Tilley WD. PC-3 cells with enhanced androgen receptor signaling: a model for clonal selection in prostate cancer. *The Prostate* (In Press, 2004).

Two sublines of the human prostate cancer cell line, PC-3, which is widely used as a model of prostate cancer progression, have been reported: PC-3^{AR-} that do not express AR, and PC-3^{AR+} that have measurable AR RNA but little protein. We assayed the genotype, karyotype, AR expression and physical characteristics of the two PC-3 sublines, and compared their ability of elicit a transactivation response from ectopic AR in the presence and absence of specific AR coregulators. PC-3^{AR-} and PC-3^{AR+} cells are genotypically and karyotypically similar, but exhibit salient differences in their morphology, growth rate and expression of AR RNA. Whereas endogenous AR expression in PC-3^{AR+} cells does not result in sufficient protein to confer androgen responsiveness in culture, ectopic AR consistently elicited a much greater transactivation response in PC-3^{AR+} than in PC-3^{AR-} cells, without altered sensitivity to activation by native ligand or AR coregulators including GRIP1, BRCA1 and Zac1. Moreover, phenotypic differences of AR variants implicated in prostate cancer susceptibility and progression were only observed in PC-3^{AR+} cells. Higher levels of known AR coregulator proteins detected in PC-3^{AR+} compared with PC-3^{AR-} cells likely contribute to these differences. These studies provide new evidence that the androgen-signaling axis can be sensitized in prostate cancer cells, and have important implications for the analysis and interpretation of AR structure and function in *in vitro* cell systems.

7) Buchanan G, Yang M, Cheong A, Harris JM, Irvine RA, Lambert PF, Moore HL, Raynor M, Neufing PJ, **Coetzee GA**, Tilley WD. The role of the androgen receptor polyglutamine tract: structural and functional consequences of glutamine tract variation and evidence for an optimal repeat length. *Hum Mol Genet* (under review and provisionally accepted, 2004).

The androgen receptor (AR) gene contains a polymorphic trinucleotide repeat region, $(CAG)_n$ in its amino-terminal transactivation domain (NTD) that encodes a polyglutamine (polyQ) tract in the receptor protein. While the length of the (CAG)n repeat ranges from 6-39 in healthy individuals, variations in repeat length both within and outside the normal range are associated with disease, including impaired spermatogenesis and Kennedy's disease, and with the risk of developing breast and prostate cancer. While an inverse relationship between polyQ tract length and transactivation potential of the receptor may result from altered recruitment of accessory proteins, the molecular mechanisms by which polyQ length modulates these effects has not been elucidated. In this study, we provide detailed characterization of a somatic AR gene mutation detected in a human prostate tumor that results in interruption of the polyQ tract by two non-consecutive leucine residues (AR-polyQ2L). Compared to wtAR, AR-polyQ2L exhibits disrupted inter-domain communication (N/C interaction) and an associated lower protein level, but paradoxically has markedly increased transactivation activity. Molecular modeling and the response to cofactors indicates that AR-polyQ2L provides a more stable platform for the recruitment of accessory proteins than wild-type AR. Analysis of the relationship between polyQ tract length and AR function revealed a critical size (Q16-Q29) for maintenance of N/C interaction. That up to 99% of AR alleles in different racial-ethnic groups encode a polyglutamine tract within this range suggests that positive evolutionary selection may act to preserve N/C interaction as an essential component of androgen induced AR signaling.

Key Research Accomplishments

A total of 12 publications emanated from work done supported by this grant and key research accomplishments were summarized in four reviews and one editorial:

1. Buchanan G, Irvine RA, Coetzee GA, Tilley WD. Contribution of the androgen receptor to prostate cancer predisposition and progression. *Cancer Metastasis Rev* 20:207-223, 2001.

2. Coetzee GA, Irvine RA. Size of the androgen receptor CAG repeat and prostate cancer: does it matter? (Editorial) *J Clin Oncol* 20:3572-3573, 2002.

3. Tilley WD, Buchanan G, Coetzee GA. Androgen receptor signaling in prostate cancer. Hormones, Genes and Cancer (Edited by Henderson, Ponder & Ross) Oxford University Press, pp 288-315, 2003.

4. Clark PE, Irvine RA, Coetzee GA. The AR CAG repeat and prostate cancer risk. *Methods Mol Med* 81:255-266, 2003.

5. Kim J, Coetzee GA. Androgen receptor activity at the PSA locus. *J Cell Biochem* (invited review to be published under "Perspectives", under preparation, 2004).

Reportable Outcomes

The work was published in 12 papers as outlined above, all of which are reportable.

Conclusions

The single main conclusion from the present studies (supported by the present grant) is that the AR plays a vital role in prostate cancer progression during androgen-independent phases of the disease as a consequence of androgen ablation therapy. The AR forms a nexus from which many mechanisms of prostate cancer growth control participate to yield cells with a growth advantage. It is obvious that targeting the AR (rather than the ligand- hormone) should yield therapies for advanced prostate cancer that are much better than present treatment regimens. A negative conclusion was that somatic mutations in the AR do not contribute to tumor growth in primary prostate tumors not obtained after hormone ablation therapy.

References

Balk, S. (2002). "Androgen receptor as a target in androgen-independent prostate cancer." Urology **60**(3 Suppl 1): 132-138.

Buchanan, G., N. M. Greenberg, et al. (2001). "Collocation of androgen receptor gene mutations in prostate cancer." Clin Cancer Res **7**(5): 1273-81.

Buchanan, G., R. A. Irvine, et al. (2001). "Contribution of the androgen receptor to prostate cancer predisposition and progression." Cancer Metastasis Rev **20**(3-4): 207-23.

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Feldman, B. J. and D. Feldman (2001). "The development of androgen-independent prostate cancer." Nat Rev Cancer **1**(1): 34-45.

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Kim, J., L. Jia, et al. (2003). "Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor." Nucleic Acids Res **31**(23): 6741-7.

Park, J. J., R. A. Irvine, et al. (2000). "Breast cancer susceptibility gene 1 (BRCAI) is a coactivator of the androgen receptor." Cancer Res **60**(21): 5946-9.

Xue, W. M., G. A. Coetzee, et al. (2001). "Genetic determinants of serum prostate-specific antigen levels in healthy men from a multiethnic cohort." Cancer Epidemiol Biomarkers Prev **10**(6): 575-9.

Appendices

Copies of all the papers that emanated from this grant and referred to above are attached.

Project C: Cellular and Molecular Markers of Prostate Cancer Progression
PI: Richard Cote, M.D.

Introduction

Prostate cancer is a highly heterogeneous disease with an unpredictable course. Although the epidemiology and etiology of prostate cancer is largely unknown, it is a disease with extraordinary racial-ethnic variation in incidence, mortality, and survival. African-American men have by far the highest rates of prostate cancer in the world, whereas Asian men native to China, Japan and Korea have the lowest. Even for prostate cancers presenting at a specific stage, African-Americans have substantially worse survival, whereas Asian-Americans appear to have substantially better survival than whites including Hispanics. Indeed, a recent report shows that even in an equal-access medical care setting, prostate cancer survival for black men is poorer compared to white men, suggesting that the disease is particularly aggressive in black men.

The steps that a tumor must undergo to be invasive and metastatic (i.e. the critical factors leading to patient death) are becoming increasingly well characterized. These include:

- Loss of hormonal regulation that can also have important implications in the control of metastatic disease.
- Loss of cell cycle control: Loss of tumor suppressor function (e.g. p53, Rb, PTEN) that can have multiple effects on regulation of cell growth, angiogenesis, and the ability of a tumor to enter the cell death (apoptotic) pathway. Similarly, inactivation of cdk-inhibitors (p27, p21, p16) is expected to result in increased proliferation rates of tumor cells (as detected by PCNA, Ki67 and Topoisomerase II expression).
- Loss of growth control: In the last year a number of groups have identified loss of function of the PTEN phosphatase as a common event, particularly in advanced prostate cancer. The primary consequence of loss of PTEN function is deregulation of the PI3-kinaseAkt pathway, which is oncogenic in many tumor models. By measuring the status of this pathway at multiple levels, we will define the frequency of this change in multiple ethnic groups.
- The ability to form a new blood supply (angiogenesis), which is important in delivering nutrients and removing waste from a tumor, and also in providing a route for tumor metastasis. Loss of normal inhibitors of angiogenesis (thrombospondin-1) can lead to increased neovascularization (detected by microvessel density).
- Loss of normal cell matrix adhesion properties and cell-cell interactions (including contact inhibition), which allow tumor cells to grow past normal cell density and to break away from their primary site and form occult metastases, or overt metastases.

Body

While we originally set out with a certain set of markers that were thought to be most appropriate, we are aware that as new information arises, it is important to constantly identify new markers that will be more useful in helping us address the biovariability of tumors among members of different racial-ethnic groups and novel technologies to improve analysis capabilites. We have already identified and described two new markers to add to our test battery; Cox-2 and Caveolin-1 both of which we reported on previously. In summary, Cox-2 is an isoform of cyclooxygenase and is an enzyme that metabolizes arachidonate to prostaglandin G2 and then to prostaglandin H2. Caveolins are major structural proteins of caveolae-specialized plasma membrane invaginations that are abundant in smooth muscle cells, adipocytes, and endothelium, and mediate signal transduction activities and molecular transport.

Most recently we have been working with Dr Amy Lee from our institution. She was among the first investigators to have identified glucose-regulated protein or GRP 78 and is a world leader in research on this novel tumor marker. GRP78 is a 78,000-dalton endoplasmic reticulum lumen protein. In mammalian cells, GRP78 binds transiently to proteins traversing through the ER and facilitates their folding, assembly, and transport. As such, GRP78 is a member of a class of proteins known as molecular chaperones. Overexpression of GRP78 is believed to play a role in the progression of prostate cancer, patients with high levels of GRP78 protein may have a poorer prognosis , i.e. decreased survival time and may develop androgen independent tumors. It is thought that the induction of GRP78 proteins leads to protection and confers cell survival. Glucose starvation , acidosis and hypoxia are the characteristic microenvironments of solid tumor that are poorly vascularized. This microenvironment is thought to contribute to stress-related conditions in which GRP78 is up-regulated. We have validated the marker as an IHC marker for formalin-fixed, paraffin-embedded tissue with success and have already stained over 120 cases from this study with the protein (figure 1). The analysis of these tissues has already begun. We are currently in the process of staining the remainder of the cases with tumor tissue for analysis.

Figure 1
GRP78 expressed in a prostate cancer specimen from this study.

Since we have numerous markers to analyze, we have developed, in collaboration with George McNamara, a multi-marker technique by which we can look at up to 4 or more different markers of biologic status on a single tissue section using spectral imaging techniques. Using this technique, we can assess three to four different tumor markers on a single tissue section, thus utilizing our limited tissue resources most efficiently. Until this technique was developed and optimized for use in our laboratory we were unable to test multiple markers of interest on the limited number of slides available to us.

These new markers and novel techniques to maximize available tissue and standardize interpretation will better enable us to determine the relationship between the changes in these key biological pathways and a) race/ethnicity, b) age, and c) intermediate markers of tumor progression (tumor stage and grade). We will eventually be in a position to relate these markers to clinical outcome (survival and mortality across racial-ethnic groups).

Key Research Accomplishments

One of the major functions of this laboratory is to analyze the tissue from the submitting institutions for a variety of parameters. We are to date still receiving tumor blocks to be cut and assessed for suitability. Initially the tissue is assessed for type, (i.e resection, core biopsy or TURP). Each tissue section is then examined for the amount of tumor present and a Gleason score is given. To date we have received formalin-fixed, paraffin-embedded tissue from 507 cases of prostate cancer and entered these into the laboratory database providing them with a laboratory number. This number is linked in our database to the patient's study identification number. We have assessed 465 of these for the presence of tumor, for the percentage of tumor to normal prostate tissue and recorded the Gleason grade of the tumor in the slides provided. In most cases, sufficient tissue is available for immunohistochemical analysis. However, we have noticed a recent increase (within the past 1 year) in the number of cases received

that do not contain any tumor or have insufficient tumor for meaningful IHC analysis. Currently, the percentage with insufficient tumor is 18.5%.

In addition to the analysis for IHC suitability, we have examined the tissue and assessed its suitability for DNA extraction. On specimens that have sufficient tumor for this purpose the areas of cancer are traced out on the H&E section, then copied on the back of an unstained section and colored in red. It is primarily resection samples with greater than 5% tumor that are suitable; core biopsy specimens generally do not contain enough tumor tissue for this type of analysis. This technique is done on two unstained sections per eligible case. Slides that sufficient tumor (non-tumor tissue is outlined in blue) are sent to Drs Coetzee and Reichardt for their analysis. This technique allows their laboratory to differentially remove tumor verses non-tumor on the slide. We have identified 192 specimens that contain sufficient tumor for successful extraction. Tissue from 184 of these cases has been supplied to Dr. Coetzee (Project B) and Dr. Reichardt (Project A) for analysis.

In addition, our other major function in this study is to conduct immunohistochemical staining and analysis of specified markers. To date we have stained, reviewed and recorded the results of p27 on 389 cases, COX-2 on 357 cases, GRP78 on 111 cases and on Caveolin-1 on 75 cases. The additional markers that we still plan to assess as part of this project include factor VIII related protein for MVD and at least some of the following: bcl-2, E-cadherin p53, Rb, CD34, p21, p16, Ki67, PCNA, Topoisomerase-II and thrombospondin-1. All of these markers have been optimized by our laboratory and are ready for application. The limiting factor is available tissue per case. The multiple marker analysis by Spectral Imaging will allow us to do more markers with the limited tissue available.

Table 1

Marker	No. Cases stained	No. cases with results
p27	389	389
Cox-2	357	357
Caveolin-1	75	64
GRP-78	351	111

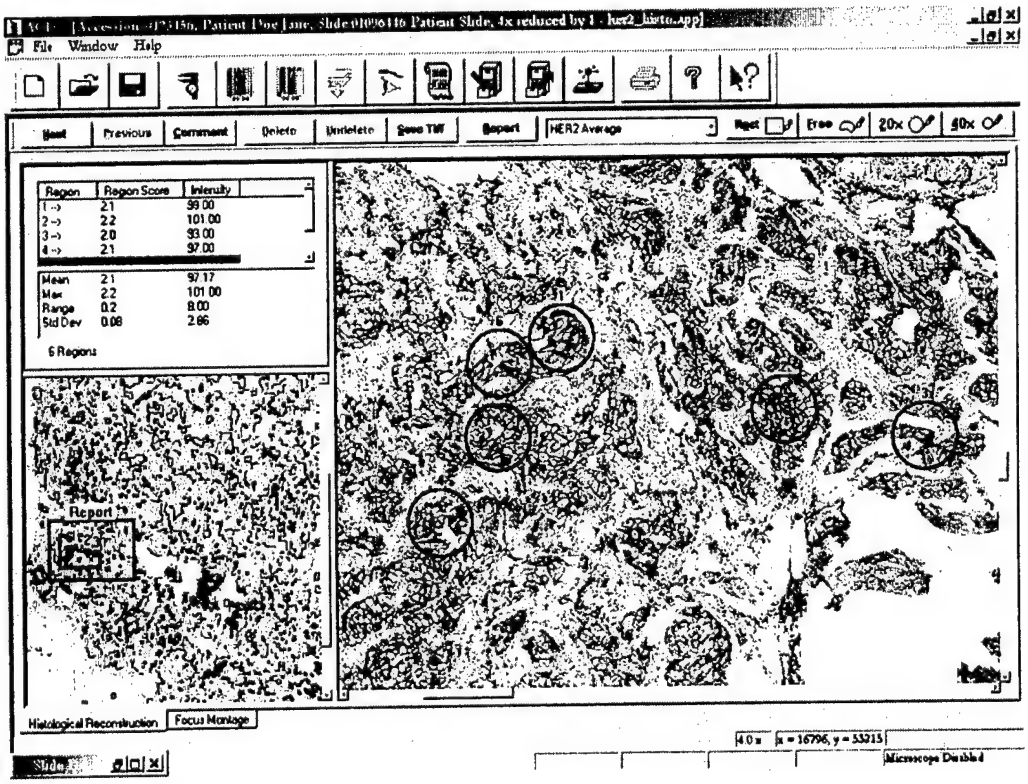
Last year we reported that we had another, novel technique for the assessment of these markers. Subjectivity and inter-observer variation may play a role in the interpretation of certain markers. This Automated Cellular Imaging System or ACIS (ChromaVision, San Juan Capistrano, CA) is intended to detect, classify, and count cells of interest based on the recognition of cellular objects of particular color, size and shape using standard laboratory cell staining procedures. We are able to modify the detection parameters to suit each individual antibody used in this study. This system can determine the percentage and intensity of immuno-positivity in specific areas. These areas are specifically chosen by the pathologist and any number of fields or size of fields that are appropriate can be instantly analyzed once the slides are scanned in (figure 2). We have acquired color threshold software that allows us a great deal of

flexibility in utilizing this technology. We are currently in the process of assessing some of the cases stained for p27 from this study using the ACIS system. The results thus far have been promising when compared to manual reading, but certain staining patterns have proven difficult for the ACIS to correctly interpret (i.e., strong hematoxylin counter-staining). We will be receiving updated software that has the potential of solving these problems within the next two weeks. We will continue with the side-by-side manual ACIS readings for all cases with sufficient tumor. The ACIS results are secondary results; the reported results will be the validated and tested manual interpretations. Nonetheless, the ACIS data will be compared with standard techniques for its potential use as a first-line method of assessment in the future.

In addition, the ACIS is a ideal method for object quantitation of microvessel density (MVD) as determined by Factor VIII related protein IHC. Manual interpretation is difficult, time consuming and is subject to inter-observer variability, which makes this capability particularly import for this study. As soon as the current markers have been completed, we will stain all cases with sufficient tumor burden for MVD as stated in our original specific aims.

We remain blinded to all clinical and ethnic information regarding these patient samples, and we will remain so until the final interpretations have been reached for the markers in question as not to bias our interpretations.

Figure 2 Example of automated IHC slide analysis by ACIS



Reportable Outcomes

All outcomes are reportable and analyses and manuscript preparation are ongoing.

Conclusions

This study is a molecular epidemiologic study designed to study prostate cancer progression. It has taken an innovative approach to develop and apply novel biologic markers of prostate cancer progression, and we are continuing to add novel techniques that will increase the information we can obtain from this study. We are applying novel technologies to this study that were not even available to us when the project was proposed. We are continuing to receive more case samples and are continuing to make significant progress in our goals to assess multiple, significant markers of disease progression.

Core: Epidemiology Core
Director: Brian E. Henderson, M.D.

Introduction

This study was proposed to look at differences in tumor behavior (molecular and cellular behavior) observed in prostate cancer arising in men of different racial groups. The specific aims were to: (1) identify and contact incident prostate cancer patients diagnosed among participants in the Hawaii-Los Angeles multiethnic cohort (MEC) study to obtain signed tissue release forms, (2) secure formalin-fixed tissues on these individuals; to process these samples; and to distribute these samples to laboratories involved, and (3) develop and implement data forms to record laboratory results and histologic reviews; to conduct data management activities including data entry and editing.

Body

During this study in Los Angeles, we continuously identified newly diagnosed African-American and Latino-American prostate cancer cases in the multi-ethnic cohort. 1412 men were identified and contacted by mail, and in some cases, by phone and asked to sign tissue release forms. These consents were approved by the University of Southern California IRB office and sent to the men identified as having prostate cancer through follow-up linkages with our SEER cancer registry. 698 men (348 African-American and 350 Latinos) signed the forms and returned them to us by mail. Although funding has ended we are continuing the process of calling the non-respondents to encourage them to sign and return the consent forms. Seventy-seven subjects have died and we are trying to secure tissue release forms signed by next-of-kin. Sixty-three subjects have refused participation.

We provided 496 tissue release request forms to date to the Tissue Procurement Core Resource at USC/Norris Comprehensive Cancer Center. We have completed the process of requesting tissue subjects. We have received tissue on 166 African-Americans and 165 Latinos to date. All have been forwarded to Dr. Richard Cote's (Project C) laboratory for processing and distribution.

We have received 191 tissues samples from the University of Hawaii; 98 from Caucasians and 79 from Japanese-Americans; 4 others are African American, 2 are Hawaiian, 4 are Filipino and 4 are Hispanic. Thus we have obtained and processed 522 tissue samples to date, forming one of the largest population based multiethnic tissue banks on prostate cancer in existence.

Key Research Accomplishments

None. This is a Core resource to support the three Projects.

Reportable Outcomes

None

Conclusions

After working out the complex logistics to run this core, it functioned very efficiently. Prostate cancer patients were routinely identified among all four racial ethnic groups in this study and signed tissue releases were obtained. Tissue procurement and processing has gone well in African-Americans and Latinos and, after delays due to IRB issues, tissue procurement proceeded within Japanese and Whites. Although funding has ended we plan to continue to process tissues from both LA and Hawaii on those patients already in process. We expect to approach a final tissue bank size of at least 550 patients.

Identification and Characterization of Somatic Steroid 5α-Reductase (*SRD5A2*) Mutations in Human Prostate Cancer Tissue

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Running Title: Somatic SRD5A2 Mutations in Prostate Cancer

Abstract

Prostate cancer is a very common disease in industrialized countries and it is known to be androgen-dependent. The human *SRD5A2* gene encodes the prostatic (or type II) steroid 5α-reductase, which catalyzes the irreversible conversion of testosterone to dihydrotestosterone (DHT), the most active androgen in the prostate. We have sequenced the entire protein coding region of this locus in 30 microdissected prostate adenocarcinomas. We identified a total of 17 *de novo* amino acid substitutions in 13 of these tumors. We also identified 6 additional silent substitutions. In total, 18 out of 30 (60 %) of the tumors examined had *de novo* somatic substitutions in the prostatic steroid 5α-reductase coding region. We also characterized all of the SRD5A2 missense substitutions biochemically and pharmacologically, using three 5α-reductase inhibitors, including finasteride. The biochemical parameters of the distinct 5α-reductase missense substitutions varied substantially. We note that two out of the three recurrent SRD5A2 missense substitutions increased 5α-reductase *in vitro* activity, while the third one is essentially neutral. These findings are consistent with a role for increased DHT levels in the prostate through increased activity of the SRD5A2 locus in prostate cancer progression, in a subset of patients. Our pharmacologic studies also reveal substantial variability for each 5α-reductase inhibitor. These data, therefore, should be taken into account in both prevention as well as therapeutic trials of prostate cancer utilizing 5α-reductase inhibitors.

Introduction

Prostate cancer is a very common disease in the industrialized world: 220,900 men will be newly diagnosed and 28,900 men are predicted to die of the disease in the US this year alone (Jemal *et al*, 2003). Prostate cancer is dependent on androgens for growth, and we have previously proposed that variations in androgen metabolism may affect a man's risk of this disease (Ross *et al*, 1998). We have reported evidence that increased intraprostatic androgen metabolism, particularly through the enzyme steroid 5α–reductase, may play an important role in predisposition to prostate cancer (Ross *et al*, 1992; Makridakis *et al*, 1999). Steroid 5α–reductase catalyzes the conversion of testosterone to dihydrotestosterone (DHT), the most potent androgen in the prostate (e.g. Cheng *et al*, 1993). Thus, genetic variants encoded by the SRD5A2 gene and other androgen-metabolic enzymes may play an important role in predisposition to prostate cancer (Ross *et al*, 1998). We have also reported data on somatic events at the *SRD5A2* locus (Akalu *et al*, 1999). Thus, this gene may play a role both in predisposition and in progression of prostate cancer.

Finasteride is a specific competitive inhibitor of the SRD5A2 gene product (Stoner 1996). The recent report of the PCPT (prostate cancer prevention trial) showed that treatment of men 55 years of age or older with finasteride resulted in a significant decrease in the rate of prostate cancer measured over a seven year period (Thompson *et al*, 2003), suggesting that finasteride prevents or delays the appearance of prostate cancer. However, finasteride treatment also resulted in a significant increase in the incidence of

prostate tumors of high grade Gleason (specifically grade 7 or higher; Thompson *et al*, 2003).

We report the identification of ten *de novo* SRD5A2 missense substitutions in somatic prostate cancer tissue. Four of these amino acid substitutions produce significantly more DHT in *in vitro* enzyme assays. Some of these missense mutations were also recurrent. This observation suggests that these SRD5A2 somatic mutations may play an important role in prostate cancer progression and may explain the unexpected finding of an increase in high grade prostate cancer in the PCPT (described by Thompson *et al*, 2003).

Results

We examined the entire protein-coding region of the *SRD5A2* gene for somatic (or *de novo*) mutations by microdissection of prostate cancer tissue followed by automated DNA sequencing of the isolated tumor DNA. We have identified a total of 18 amino acid (or missense) substitutions in 13 of 30 tumors (Fig. 1). Three of these missense substitutions were recurrent (Table I), including the most common one, A49T (alanine-49 to threonine), which has also been found to be involved in prostate cancer predisposition (Makridakis *et al,* 1999). We found an additional 6 polymorphic substitutions in 7 tumors (Fig. 1 and data not shown). One of these polymorphisms, C2247T (cytocine-2247 to thymine), is also recurrent (it was found in two tumors, Table I). A total of 18 tumors had acquired additional somatic changes at the *SRD5A2* locus (Table II and data not shown). Thus, about 60 % of the tumors examined show evidence of *de novo* somatic substitutions in the *SRD5A2* coding region.

Parallel examination of the corresponding peripheral blood lymphocyte (i.e. constitutional or "germline") DNA was also performed. These analyses failed to detect any of these novel substitutions found in tumor tissue, except for tumor sample 7 (Table II). Constitutional DNA of that sample was already heterozygous for the A49T missense mutation (Table II). This mutation, however, was essentially homozygote in the tumor tissue since the intensity of the mutant exceeded 89 % (Table II). Therefore, an additional somatic event must have occurred in this tumor at this particular site. Interestingly, we detected two additional somatic events in this tumor sample, an amino acid substitution (A248V) and a polymorphism (C2247T) (Fig. 1, Table II and data not shown).

Analysis of the distribution of the somatic SRD5A2 missense substitutions by tumor tissue reveals that four tumors have prevalent (more than 50 % mutant) missense substitutions in the SRD5A2 gene (Table II). In three out of the four cases the prevalent missense substitution is the A49T (Table II). The A49T is also the most prevalent missense substitution among tumors than have another missense substitution in the SRD5A2 gene, in addition to the A49T (Table II). Table II also reveals that the majority of the tumor samples that have missense substitutions also display previously identified somatic events at the SRD5A2 locus (five of those samples had microsatellite instability and two loss of heterozygosity). Thus the SRD5A2 gene is a common target for multiple somatic events in prostate cancer tissue.

We then analyzed the biochemical characteristics of each of the variant 5α-reductase proteins after transient expression of the respective reconstructed clones in mammalian cells followed by *in vitro* enzyme assays (Table III). Three classes of missense substitutions are evident (Table III): i) those that result in increased activity compared to wild-type protein, evidenced by the increased apparent V_{max} (V3I, A49T, F118L, and A248V), ii) those that display roughly normal (or "wild type") activity (V63M, G183D and L226P), and iii) those that result in decreased activity compared to the wild type protein (V189A, G191E, and L221P). For each missense substitution, changes in the apparent V_{max} are accompanied by changes in the apparent K_m for either testosterone or NADPH, or both (Table III). Interestingly, most of the missense substitutions appear to increase the K_m for testosterone, including all of those that increase the apparent V_{max} (Table III). All of the missense substitutions display optimum activity at the optimum pH for the wild type protein (pH 6), with the exception of the

L221P (leucine-221 to proline) substitution, which has an increased optimal pH of 6.5 (Table III).

Characterization of the apparent inhibitory constant (K_i) for three competitive 5α-reductase inhibitors (finasteride, dutasteride and PNU-157706; Makridakis *et al*, 2000) indicates that there is significant pharmacogenetic variation compared to the wild type protein for most of the somatic SRD5A2 missense substitutions (Table IV). The apparent K_i for the selective 5α-reductase type II inhibitor finasteride (Stoner, 1996) varied 36-fold, from 5-180 nM (Table IV). The variation of the apparent K_i for PNU-157706 (di Salle *et al*, 1998) was about 16-fold, ranging from 1.1-18 nM (Table IV). The most significant variation of the apparent K_i though was for dutasteride (Frye *et al*, 1998), which varied 85-fold, spanning the range from 1.1-93 nM (Table IV).

Discussion

We report here common *de novo* somatic mutations in the SRD5A2 (prostatic or type II steroid 5α–reductase) gene coding region in microdissected prostate cancer tissue. Specifically, 18 out of 30 (or about 60 %) of the prostate tumors examined show evidence of missense substitutions and silent mutations at the locus of interest. This figure is in line with the previously reported genomic (specifically microsatellite) instability and LOH (loss of heterozygosity) mutations at the same gene in the same samples (about 57%; Akalu *et al*, 1999). In addition, the majority of the SRD5A2 missense substitutions were present in samples that were previously shown to have either microsatellite instability or LOH at the SRD5A2 locus (Table II). Therefore, the SRD5A2 gene is a common target for multiple somatic events in prostate cancer tissue.

Three of the SRD5A2 missense substitutions and one of the polymorphisms were recurrent (Table I). Moreover, preliminary data suggests that the two most common missense substitutions, A49T and V63M (Table I), are also present in an independent data set of prostate tumor tissues (the A49T mutation was recurrent again; data not shown). It is of special interest that the most common somatic event found, the A49T mutation, was more prevalent than the normal allele (more than 50% mutant) in most of the tumors that contained it (three out of five tumors; Table II). This mutation has been previously shown to be associated with prostate cancer predisposition, and it is known to increase 5α-reductase activity *in vitro* (Makridakis *et al*, 1999). Thus, the A49T mutation in the SRD5A2 gene may contribute to the development as well as the progression of prostate cancer, through increased DHT production.

Some of the prostate tissues examined had more than one somatic SNPs and one sample (sample 7) had three (the A49T, A248V and C2247T SNPs; Fig. 1, Table II, and data not shown). The latter two SNPs were present at much lower levels than the A49T mutation in this tumor sample (Table II). These data suggest either multiple somatic mutation events in this sample or a large-scale rearrangement. We favor the former hypothesis since we detected microsatellite instability previously in this tumor (Table II) but no LOH.

Biochemical analysis of the SRD5A2 missense substitutions we identified *in vitro* suggests that four of these substitutions significantly increase steroid 5α-reductase activity (by increasing the apparent V_{max}), three are neutral, and three decrease steroid 5α-reductase activity (Fig. 2 and Table III). Moreover, two out of the three recurrent SRD5A2 somatic missense substitutions, A49T and A248V, significantly increase steroid 5α-reductase activity, while the third, V63M, is essentially neutral (Fig. 2 and Tables I and III). Thus none of the recurrent SRD5A2 somatic missense substitutions result in decreased steroid 5α-reductase activity (Fig. 2 and Tables I and III). Both the A49T and the A248V somatic mutations display significantly higher Vmax/ Km ratios, a measure of catalytic efficiency (Nelson and Cox, 2000) than the normal enzyme (data not shown). Therefore, most recurrent SRD5A2 somatic missense substitutions result in increased catalytic efficiency. In addition, the most common missense substitution (A49T; Table I) is also the mutation that results in the highest steroid 5α-reductase activity *in vitro* (Fig. 2 and Table III) and is the most prevalent mutation in each of the tissues that it is present (Table II). Taking into account the recurrent mutations, about 20% of the prostate tumor samples have somatic SRD5A2 mutations that significantly activate steroid 5α-reductase,

while only 7% have SRD5A2 mutations that reduce enzyme activity significantly. These observations are consistent with the hypothesis that increased DHT levels are important for prostate tumor progression. The small number of tumors that have inactivating steroid 5α-reductase mutations may contain additional mutations in other androgen metabolic genes so that the overall result is stimulation of the androgen receptor signaling pathway, despite lower steroid 5α-reductase activity. Future studies on other androgen metabolic genes will address this hypothesis.

The majority of the somatic SRD5A2 missense substitutions that affected the apparent K_m for testosterone (Table III) also affected the apparent K_i for all three 5α-reductase inhibitors, finasteride, dutasteride and PNU157706 (Table IV). This finding is consistent with the fact that all of these drugs are competitive inhibitors of 5α-reductase (Stoner 1996, di Salle *et al,* 1998, Frye *et al,* 1998). All of the somatic SRD5A2 missense substitutions analyzed significantly modified the apparent K_i of the normal protein for all three steroid 5α-reductase inhibitors examined (Table IV). PNU157706 was the steroid 5α-reductase inhibitor that exhibited the lowest apparent K_i for most of the SRD5A2 missense substitutions (Table IV). The distribution of the apparent K_i for the distinct SRD5A2 missense substitutions varied substantially, from 16-fold (for PNU157706) to 84-fold (for dutasteride) (Table IV). This finding is also true if one focuses only on the recurrent somatic SRD5A2 mutations (Tables I and IV), or on the naturally occurring SRD5A2 missense substitutions reported previously (Makridakis *et al*, 2000). This significant pharmacogenetic variation at the SRD5A2 locus should be taken into account when using competitive steroid 5α-reductase inhibitors for the treatment or prevention of prostate cancer. For example, the Prostate Cancer Prevention Trial (PCPT) recently

reported that finasteride treatment in men followed over seven years resulted in a significant decrease in the overall incidence of prostate cancer, but also in a significant increase in the incidence of high-grade prostate tumors (Thompson *et al*, 2003). Given the pharmacogenetic variability for finasteride presented here and elsewhere (Makridakis *et al*, 2000) for both constitutional DNA SNPs and somatic SRD5A2 mutations, future trials such as the PCPT should consider genotyping men (especially men that took finasteride and developed high-grade prostate tumors) for SRD5A2 mutations. This conclusion is also strengthened by the fact that two out of the three recurrent somatic missense substitutions increased the K_i for finasteride (Table IV). Thus, constitutional and somatic genotypes may be critical for finasteride efficacy.

The somatic missense substitutions are distributed throughout the protein-coding region of the human SRD5A2 gene (Fig. 1). However, there appears to be a clustering of missense substitutions particularly in the carboxy-terminal third of the protein, specifically codons 183-248 (Fig. 1). This part of the steroid 5α-reductase enzyme has been proposed to be involved in substrate and cofactor binding (Wigley *et al.* 1994; Makridakis *et al*, 2000). We also note the relative rarity of substitutions of any kind in the central portion of the *SRD5A2* protein, another observation previously made by us but not others (Wigley *et al.* 1994; Makridakis *et al*, 2000). We suspect that this difference may be due to the different phenotypes investigated by Russell and colleagues, male pseudohermaphroditism (cf. Wigley *et al.* 1994) a rare autosomal recessive disorder which results in mutations that severely impair enzymatic activity, and us who are interested in mutations and polymorphisms in prostate cancer that presumably activate steroid 5α–reductase (e.g. Makridakis *et al*, 1999; Makridakis *et al*, 2000). Thus male

pseudohermaphrodistism mutations are loss of function mutations (Wigley *et al.* 1994) while activating mutations in prostate cancer should be gain of function mutations (Makridakis *et al*, 1999). Analysis of the apparent binding constants for the distinct missense substitutions (Table III) can provide information about the number of domains that comprise the binding pocket of the prostatic steroid 5α-reductase, a protein that has never been purified. Only missense substitutions confined in the carboxy-terminal third of the protein affect the apparent K_m for the NADPH cofactor (Table III and Fig. 3). The binding site for the substrate (testosterone) though appears to be bipartite: both amino-terminal and carboxy-terminal domains are important for binding (Table III and Fig. 3). These findings confirm earlier observations based on SRD5A2 missense substitutions found on constitutional DNA (Makridakis *et al,* 2000). However, data reported here significantly expands the extent of the testosterone-binding site for this enzyme, at least based on its primary (linear) structure (Fig. 3).

Common karyotypic abnormalities have been reported on the short arm of chromosome 2 in prostate cancer tissue and cell lines (Brothman *et al,* 1990). Interestingly, the chromosomal location of the *SRD5A2* gene is in chromosome band 2p23 (Thigpen *et al,* 1992). Therefore, it is possible that the chromosomal abnormalities identified previously (e.g. Brothman *et al,* 1990) relate in part with the somatic mutations we identified in the SRD5A2 locus (Akalu *et al,* 1999 and this report). We note that another study that utilized Single Nucleotide Polymorphic (SNP) arrays for the analysis of LOH from laser capture microdissected prostate cancer samples did not present any data on chromosome 2 (Lieberfarb *et al*, 2003).

Somatic mutations in prostate cancer have been of intense interest to researchers (e.g. Gottlieb *et al.* 1999, Brothman *et al,* 1990). Previous investigations, however, have never focused specifically on androgen-metabolic genes. In fact, there are only two publications addressing such genes to our knowledge (Akalu *et al,* 1999, Macoska *et al,* 1992). These reports investigated the type I steroid 5α-reductase gene (SRD5A1) by Southern blotting alone (Macoska *et al,* 1992) and the type II steroid 5α-reductase (SRD5A2) gene by PCR analysis of a single dinucleotide repeat (Akalu *et al,* 1999). Thus, this report is the first to systematically investigate the entire coding sequence of an androgen-metabolic gene for somatic mutations in prostate cancer. We propose that these genes are likely to be important targets in tumor progression since the disease is androgen-dependent at least early in its development (Cheng *et al,* 1993). Furthermore, the androgen receptor (AR) gene is also mutated in some prostate cancer tissues (e.g. Gottlieb *et al,* 1999, Visakorpi *et al,* 1995). Thus the SRD5A2 gene as well as other androgen-metabolic loci together with the AR may play an important role in tumor progression. Systematic analyses of such genes may identify valuable prognostic markers and offer the opportunity for rational intervention.

Most of the studies that compared the SRD5A2 mRNA levels between normal human prostate and prostate cancer have reported decreased SRD5A2 gene expression (Luo *et al,* 2003). To reconcile these findings with the documented androgen dependence of prostate cancer in all disease stages but the metastatic and the success of finasteride in the PCPT cancer prevention trial, we propose that somatic SRD5A2 gene mutations that activate 5α-reductase may be common in prostate cancer tissue.

The large number of *de novo* mutations identified enables us to analyze the molecular nature of the somatic events occurring in those prostate tumors. The vast majority of those somatic events are transitions that occur at a nine-nucleotide purine-rich motif (manuscript in preparation). These observations suggest that the somatic SRD5A2 mutations may be generated through a distinct molecular mechanism. It may also be of technical interest to note that in general both the sequencing and SSCP methods gave similar quantitation of all somatic mutations (Table II). This finding suggests that both methods can accurately detect and quantitate such mutations. Preliminary analyses of the distribution of the SRD5A2 mutations by stage indicates that the majority of the SRD5A2 somatic events occurred rather early during prostate cancer progression compared to the overall stage distribution of the samples (Table II and Akalu *et al,* 1999).

In summary, we report here both missense and silent somatic (*de novo*) substitutions in the protein coding region of the SRD5A2 gene (encoding the prostatic steroid 5α-reductase) in about 60 % of prostate tumors examined. Some of these substitutions were recurrent. Kinetic analysis of the missense SRD5A2 substitutions revealed significant pharmacogenetic and biochemical variation. The majority of the recurrent missense SRD5A2 substitutions resulted in significantly increased apparent V_{max} *in vitro*. These common SRD5A2 somatic mutations may be relevant to prostate tumor progression and their biochemical and pharmacogenetic consequences may help in individualized cancer treatment (and perhaps even prevention).

Materials and Methods

DNA Samples from Tissue

Samples and stage information are as in Akalu *et al*, 1999. Briefly, tumor prostate and peripheral blood samples were obtained from 30 patients who underwent radical prostatectomy between 1988 and 1995 at the USC/ Norris Comprehensive Cancer Center. These patients were heterozygous for the $(TA)_n$ marker in the SRD5A2 gene (Akalu *et al*, 1999). Tumor staging was done according to the TNM classification system (Schroder *et al*, 1992).

Mutation Identification

DNA was extracted from microdissected tissue exactly as described above (see also Akalu *et al*, 1999, Akalu and Reichardt, 2000). Primers for PCR (polymerase chain reaction) amplification of *SRD5A2* exons 1 through 5 were described previously (Makridakis *et al*, 1997). An additional primer was used to amplify the upstream part of exon 1 using the forward primer 5'-GAAGCCCTCCGGCTACGGG-3' and the reverse primer 5'-TGCACTGGGCGCCCGCAAG-3'. PCR products were obtained by thermal cycling using a RoboCycler Gradient 40 system (Stratagene; La Jolla, CA) under the following conditions: an initial denaturation step at 94 ^{0}C was performed for 2 min followed by 43 cycles of denaturation at 95 ^{0}C 1 min, annealing at 60 ^{0}C for 1 min, and 2 min extension at 72 ^{0}C. All PCR products were then extended at 72 ^{0}C for 5 min. PCR products were analyzed by SSCP as described previously (Makridakis *et al*, 1997). Sequencing analysis was done using the ABI PRISM™ Dye Terminator Cycle

Sequencing Ready Reaction kit (Perkin Elmer; Foster City, CA). Sequencing reactions were run, analyzed and quantitated on an ABI PRISM 377 DNA Sequencer (Foster City, CA) using software provided by ABI. Signal intensities of SSCP bands were measured by the ImageQuant software (Sunnyvale, CA).

Similar analyses of the matched constitutional ("germline") DNA were performed to verify that the somatic mutations in fact occurred *de novo*.

Mutation Characterization

Individual mutations were reconstructed in the SRD5A2 cDNA mammalian expression vector pS303-5αR2 (originally obtained from Dr. D. Russell, Southwestern Medical College, Dallas, TX; see Makridakis *et al* 2000), by site-directed mutagenesis using the QuickChange kit (Stratagene, San Diego, CA, USA) with custom primers (Invitrogen, Carlsbad, CA, USA). The distinct SRD5A2 cDNAs were electroporated in *cos7* cells together with a β-galactosidase control plasmid, and the respective protein extracts were collected 48 hours later, by sonication. β-Galactosidase assay was then performed to normalize the extracts for differences in transfection efficiency. Normalized extract amounts were then used for 5α-reductase activity assay, as described (Makridakis *et al* 2000). Briefly, a mixture containing 5 mM NADPH, 0.2 μM [^{14}C]-testosterone, various amounts of unlabeled testosterone and 0.1 M Tris-citrate buffer (pH 6), was added to the normalized protein extracts on ice. The reactions were placed at 37 ^{0}C for 10 minutes. Subsequently, the reactions were stopped with methylene chloride, and after extraction of the organic phase, steroids were dried, redissolved and separated by thin-layer chromatography (TLC) (Makridakis *et al* 1999 and 2000). Dried TLC plates were then

exposed on phosphorimager screens (Molecular Dynamics; Mountain View, CA, USA) and quantified on a Storm phosphorimager (Molecular Dynamics; Mountain View, CA, USA). Then, substrate to product conversion values were translated into velocity values, and plotted using Cricket Graph 1.3 (Cricket software; Malvern, PA, USA). Lineweaver-Burk plots were used to calculate V_{max} and K_m values. K_i values were calculated by similar plots after the inclusion of different amounts of inhibitors together with 1.6 μM testosterone, in 5α-reductase assays. Experimental details can be found in Makridakis *et al* 2000.

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Abbreviations

DHT	dihydrotestosterone
LOH	loss of heterozygosity
PCR	polymerase chain reaction
SNP	single nucleotide polymorphism
SRD5A2	steroid 5α–reductase type II locus
SSCP	single strand conformation polymorphism

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VARIANT	INCIDENCE	FREQUENCY
Amino Acid Substitutions		
A49T	5	17%
V63M	3	10%
A248V	2	7%
Silent Substitutions		
C2247T	2	7%

Table I: Distribution of recurrent somatic SRD5A2 substitutions in prostate tumors.

Notes: The total number of prostate tumor tissues analyzed is 30. Missense substitutions are indicated by the normal amino acid and the codon number followed by the mutant amino acid, using the one letter code. DNA substitutions are indicated by the normal base and the base number followed by the mutant base. Frequencies are approximate.

Tissue ID	*Stage*	*DNA Mutation*	*Resulting Mutation*	*Mutant (%) (SSCP)*	*Mutant (%) (Forward Sequence)*	*Mutant (%) (Reverse Sequence)*	*Previous Somatic Mutations*
1	pT2c	G750A	V3I	17	21	28	LOH
2	pT2a	G930A	V63M	11	10	29	GI
3	pT4a	G930A	V63M	15	19	32	none
4	pT2b	G930A	V63M	13	11	27	none
5	pT2	G888A T1294C	A49T F118L	40 38	53 44	29 24	none
6	pT3a	G888A G1890A	A49T G183D	71 26	78 31	44 24	GI
7*	pT3a	G888A (G888A C2285T	A49T A49T A248V	91 50 36	95 62 15	89 34) 30	GI
8	pT3a	G888A	A49T	31	38	17	LOH
9	pT3a	G888A	A49T	89	91	83	GI
10	pT3a	T1908C	V189A	41	49	47	none
11	pT3c	G1914A	G191E	38	40	33	GI
12	pT2c	T2004C C2285T	L221P A248V	100 44	100 24	100 50	none
13	pT3a	T2019C	L226P	45	43	36	none

Table II: Somatic Missense Substitutions in the Human *SRD5A2* Gene

Notes: DNA substitutions are indicated by the normal base and the base number (taken from Genbank accession number L03843) followed by the mutant base. Amino acids similarly use the one letter code and codon number. Intensities were determined once for SSCP (single strand conformation polymorphism) runs and at least three times on different sequencing runs. These data are reported as the mutant peak intensity in % of total peak intensity. Previously identified mutations (LOH being loss of heterozygosity and GI being genomic (i.e. microsatellite) instability) are from Akalu *et al*, 1999. Sample 7 was heterozygous for the A49T mutation in its constitutional DNA and is marked by an asterisk. The constitutional DNA data are, therefore, shown in parenthesis.

Variant	V_{max} nmol/m in/mg	K_m; T µM	K_m; NADPH µM	pH optimum
WT	2 (1.7-2.2)	0.6 (0.5-0.8)	8 (6-14)	6.0
V31	5	3	12	5.5-6.0
A49T	9.8	1.5	7	6.0
V63M	1.6	1.5	11	6.0
F118L	8.2	4.5	17	6.0-6.5
G183D	1.8	1.4	19	6.0
V189A	0.8	1.3	39	6.0-6.5
G191E	0.9	1	25	6.0
L221P	0.7	0.7	31	6.5
L226P	1.8	0.3	22	6.0
A248V	6.3	1.4	5	6.0

Table III: Kinetic analysis of somatic SRD5A2 missense substitutions.

Notes: Missense substitutions are indicated by the normal amino acid and the codon number followed by the mutant amino acid, using the one letter code. The experimental range for the normal (wt) type II 5α-reductase enzyme is given in parentheses.

Variant	K_i; finasteride nM	K_i; PNU nM	K_i; GG745 nM
WT	60 (52 - 72)	6 (5- 7)	17 (15 - 20)
V3I	27	3	93
A49T	180	1.1	1.1
V63M	38	10	36
F118L	5	1.7	4
G183D	45	2.6	13
V189A	90	12	23
G191E	40	18	25
L221P	57	9	14
L226P	105	5	9
A248V	85	10	13

Table IV: Pharmacogenetic analysis of somatic SRD5A2 missense substitutions.

Note: The experimental range for the normal (wt) type II 5α-reductase enzyme is given in parentheses. PNU stands for PNU157706 and GG745 is dutasteride.

Figures

Figure 1: Synonymous and nonsynonymous somatic mutations (or missense substitutions) in the SRD5A2 gene.

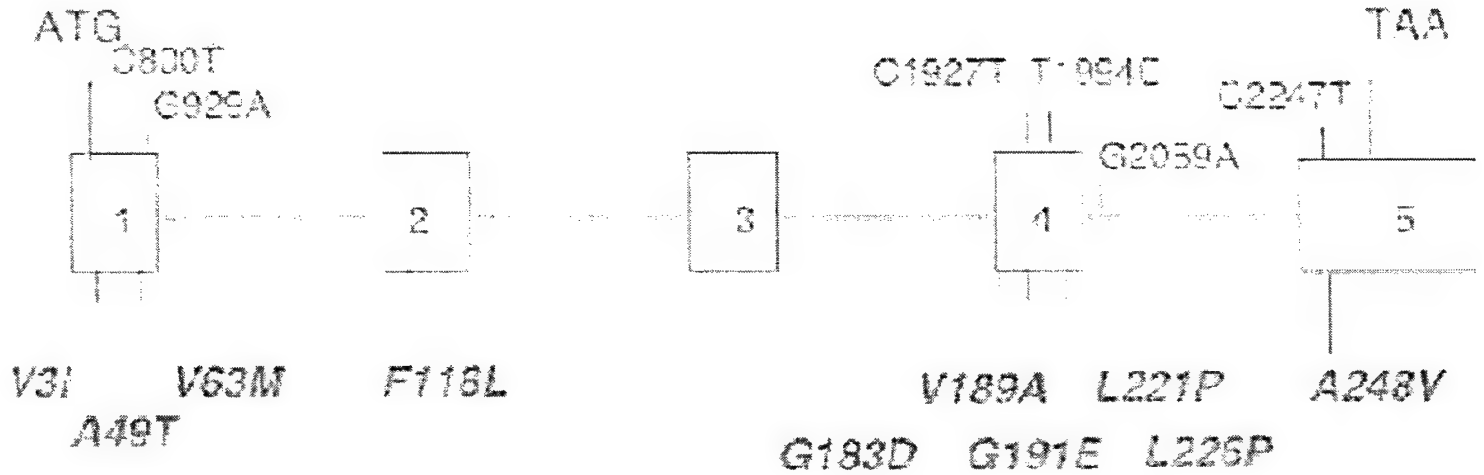
Notes: Exons are marked as numbered boxes and missense substitutions are identified below the gene by the single-letter amino acid code and the codon number. DNA substitutions are indicated above the gene by the normal base and the base number followed by the mutant base. The translation start (ATG) and stop (TAA) codons are indicated above the gene. The figure is not drawn to scale.

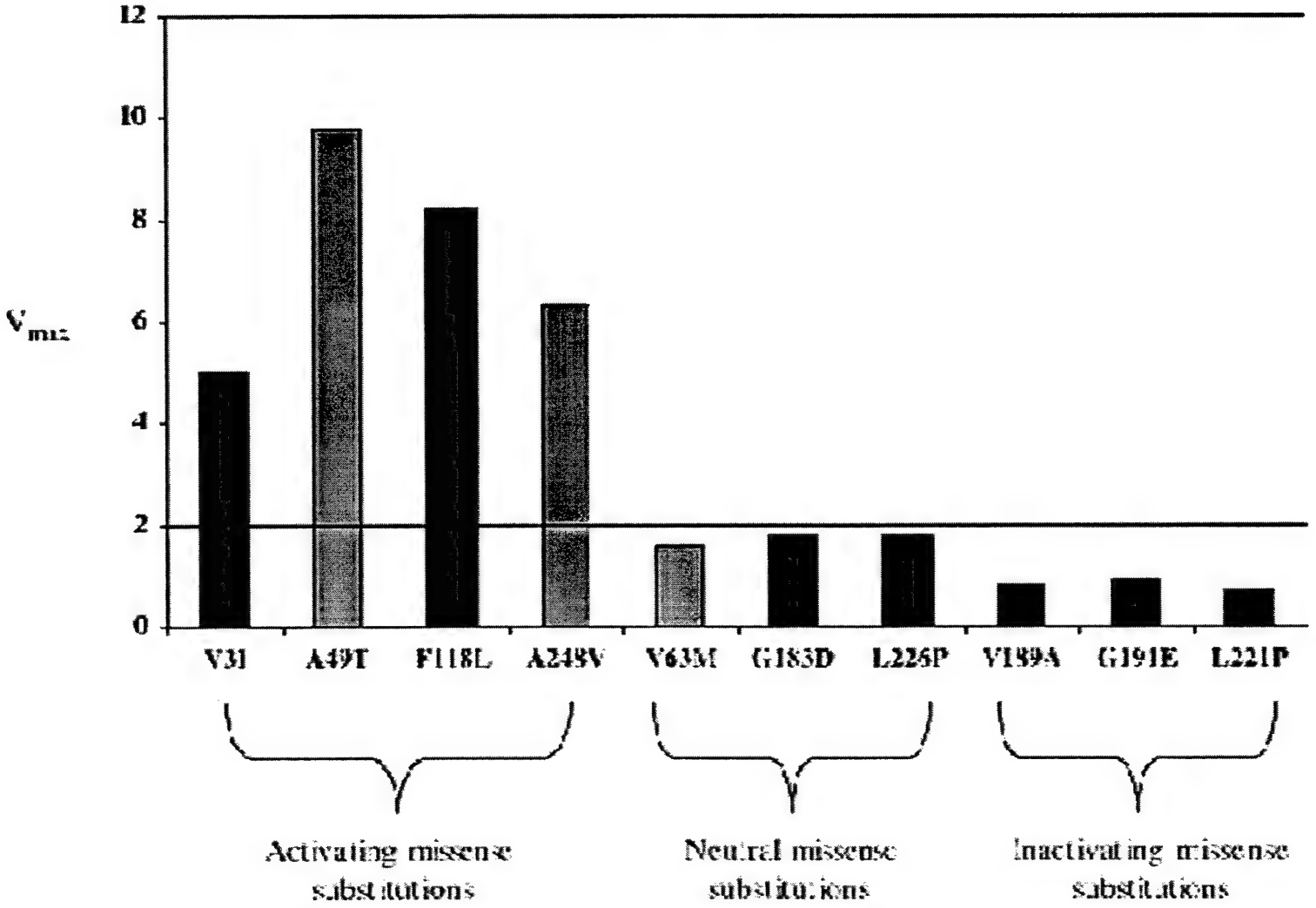
Figure 2: Comparison of the apparent V_{max} values of the somatic missense SRD5A2 substitutions.

Notes: The Y-axis indicates apparent V_{max} (nmol/ min/ mg protein). The horizontal line at 2 nmol/min/mg indicates normal (wild type) apparent V_{max}. Light colored columns indicate recurrent missense substitutions, while dark colored columns indicate missense substitutions that were only found in one prostate tumor sample. The missense substitutions are grouped according to their activity compared to wild type protein (activating, inactivating and neutral).

Figure 3: Model depicting the binding sites for the substrate (testosterone) and cofactor (NADPH) in the human type II steroid 5α-reductase enzyme.

Notes: Light colored blocks under the sequence indicate binding regions deduced from data presented in this paper, while dark colored blocks indicate binding regions reported previously in the published literature (Makridakis *et al*, 2000, Wigley *et al.* 1994). The positions of amino acids 100 and 200 are indicated as reference points.


ATG
C800T
G929A
TAA
C1927T
T1984C
G2247T
G2059A
1
2
3
4
5
V3I
A49T
V63M
F116L
V189A
G183D
G191E
L221P
L226P
A248V


12
10
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V31
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F118L
A248V
V63M
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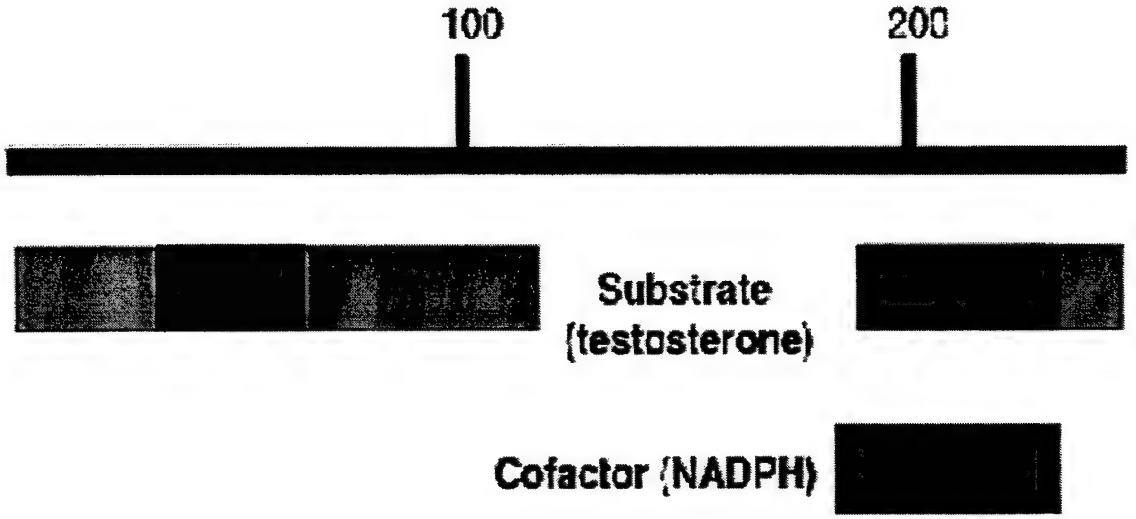
L226P
V189A
G191E
L221P
Activating missense substitutions
Neutral missense substitutions
Inactivating missense substitutions


100
200
Substrate
(testosterone)
Cofactor (NADPH)

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MOLECULAR EPIDEMIOLOGY OF ANDROGEN-METABOLIC LOCI IN PROSTATE CANCER: PREDISPOSITION AND PROGRESSION

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ABSTRACT

Purpose: We review recent molecular epidemiological data with regard to the association between several allelic variants of certain androgen-metabolic genes and the predisposition to and progression of prostate cancer.

Materials and Methods: We review recent data dealing with genetic variations in androgens and the etiology of prostate cancer.

Results: Recent molecular epidemiological data support an association between several allelic variants of certain androgen-metabolic genes and the predisposition to and progression of prostate cancer. While some of the allelic variants examined are consistently shown to be associated with increased prostate cancer risk, most of the variants show significant variability in risk.

Conclusions: A multidisciplinary attack on this problem, involving biochemistry, molecular genetics, pharmacogenetics, endocrinology and epidemiology, may be a useful paradigm in the analysis of prostate cancer and other complex human diseases. Based on the reviewed literature, we propose a guide on how and which single nucleotide polymorphisms to use in linkage and association studies of multifactorial phenotypes.

KEY WORDS: epidemiology, molecular; metabolism; androgens; phenotype; pharmacogenetics

It is estimated that 220,900 United States men will have been newly diagnosed with prostate cancer and 28,900 will have died of the disease in 2003.[1] In fact prostate cancer is the most commonly diagnosed malignancy among men in the United States.[1] Prostate cancer also represents a substantial public health problem in other industrialized nations, such as those of the European Union.[2] Androgens have a critical role in normal and abnormal prostate development. Studies of androgens and prostate cancer go back more than 60 years, and Huggins and Hodges won the Nobel Prize for their discoveries concerning hormonal treatment of prostate cancer in 1966.[3]

Prostate cancer is rare before the age of 40 years but the rate of increase thereafter is greater than that for any other cancer.[4] There is a large variation in prostate cancer rates between racial/ethnic groups in the United States. For example, in Los Angeles, black men, who have the highest prostate cancer rate in the world, have a 70% higher rate than white men, who have a substantially higher rate than Hispanic Americans, while Chinese-Americans and Japanese-Americans have roughly half the rate of white men.[5] However, Chinese and Japanese men have among the lowest prostate cancer rates in the world, that is an eighth to a twentieth the rates of United States men.[5] These epidemiological data strongly suggest a substantial genetic component to prostate cancer risk, although environmental factors are also known to have a role. Finally, there is significant evidence of more aggressive disease and less favorable outcome (eg lower survival) for black men than white men with prostate cancer.[6] These differences may be partially due to differences in socioeconomic status.

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There is a significant familial component to prostate cancer risk. In young men the familial form is most consistent with an autosomal dominant mode of inheritance.[7] However, the location and action of putative familial loci for prostate cancer remain controversial.[8]

We critically examine recent developments in the molecular epidemiological analysis of androgen-metabolic genes and the conclusions that can be drawn for prostate cancer predisposition and/or progression. Furthermore, we address the significant implications of these data for the use of single nucleotide polymorphisms (SNPs) in linkage and association studies of multifactorial diseases.

ANDROGEN ACTION

In men testosterone is synthesized in large amounts primarily by the Leydig cells of the testes.[9] Testosterone is then irreversibly metabolized intracellularly to dihydrotestosterone (DHT) in the prostate. DHT (or, much less efficiently, testosterone) is bound by an intracellular androgen receptor (AR). This complex then translocates to the cell nucleus where it activates transcription of genes with androgen responsive elements in their promoters. DHT is known to promote DNA synthesis and cell replication in the prostate. It can be inactivated in the prostate by further reduction to 3α or 3β-androstanediol. DHT levels, which are critical for prostate development, are determined by its biosynthesis, catalyzed by steroid 5α-reductase,[9,10] and degradation, initiated through reductive inactivation by the 3α and 3β-hydroxysteroid dehydrogenase enzymes.[9]

ANDROGEN RECEPTOR

The first androgen-metabolic gene studied in prostate cancer was the AR gene, presumably because it transactivates a series of genes in response to DHT binding (Appendix 1).[9] This gene has been extensively studied in humans and other mammals. A polymorphic $(CAG)_n$ trinucleotide repeat in the AR gene, which encodes a polyglutamine stretch, has been

repeatedly reported to have a role in predisposition to prostate cancer.[11] However, other studies have shown no effect of this repeat in prostate cancer.[12] This polyglutamine repeat also appears to have biochemical consequences in vitro.[13] In addition, there have been reports of many AR mutations in constitutional and somatic DNA from patients with prostate cancer.[11,14–16] However, most of the somatic mutations in the AR gene appear in advanced prostate cancer cases.

STEROID 5α-REDUCTASE

Steroid 5α-reductase (or testosterone 5α-reductase[9,10]) catalyzes the irreversible conversion of testosterone to DHT, the most potent androgen. The 2 isozymes are type I 5α-reductase encoded by the SRD5A1 gene and type II encoded by the SRD5A2 gene (Appendix 1).[9,10]

Several investigations have examined the metabolic enzyme SRD5A2 as a candidate for prostate cancer. The SRD5A2 gene was extensively screened for SNPs.[17] Substitution of 1 amino acid (or nonsynonymous SNP), the A49T variant, results in replacement of the alanine at codon 49 with threonine. The A49T missense substitution has been repeatedly reported to significantly increase the risk of prostate cancer in different populations[18–20] and increases maximal steroid 5α-reductase activity (V_{max}) 5-fold in vitro.[18] Therefore, the kinetic data provide a rational explanation for the increased risk associated with the A49T substitution. However, other epidemiological studies report a nonsignificant association between the A49T mutation and prostate cancer predisposition,[21–23] depending perhaps on the race/ethnicity of the samples.

Functional in vitro studies of SRD5A2 variants have provided critical insights and parallel in vivo findings.[18,24,25] As noted previously, the A49T substitution results in a significant increase in V_{max}, suggesting that the substantially increased risk associated with this SNP may be the result of higher intraprostatic DHT levels caused by the A49T variant. Functional data may be critical in identifying candidate SNPs suitable for epidemiological studies since an almost 200-fold variation was identified in the V_{max} of various SRD5A2 variants.[25] Therefore, variants with substantially higher (or lower) activity may be more suitable markers for molecular epidemiological association investigations than neutral polymorphisms.

The SRD5A2 locus also encodes significant (approximately 60-fold) pharmacogenetic variation for the competitive steroid 5α-reductase inhibitor finasteride.[18,25] Interestingly, finasteride has a reduced affinity for the A49T mutant enzyme in vitro. Similar variation was identified for many other SRD5A2 enzyme variants and for 2 other 5α-reductase inhibitors.[25] This significant pharmacogenetic variation should be considered when prescribing steroid 5α-reductase inhibitors for prostatic diseases. Currently, there is an ongoing chemoprevention trial of finasteride for prostate cancer involving almost 19,000 men.[26] Pharmacogenetic considerations need to have an important role in this study and future trials and treatment regimens.

In addition to affecting predisposition, the SRD5A2 gene may also be involved in prostate cancer progression, since common somatic genetic alterations in the SRD5A2 gene in prostate tumors have also been reported.[27] This dual involvement is interesting and may open new avenues of scientific research that may, in turn, result in more rational treatment of patients. Finally, involvement of this androgen-metabolic gene in prostate cancer progression may lead to new strategies for treating androgen-insensitive tumors by considering SRD5A2 and AR gene mutations.[11,14–16]

3β-HYDROXYSTEROID DEHYDROGENASE

DHT, the most active intraprostatic androgen, is inactivated through 2 reductive reactions catalyzed by 3α and 3β-hydroxysteroid dehydrogenase.[9] Therefore, 3β-hydroxysteroid dehydrogenase is critical for the regulation of intraprostatic DHT steady state levels by affecting its degradation rate. Human 3β-hydroxysteroid dehydrogenase can act on a number of steroid substrates, including DHT.[28] Enzyme activity is encoded by 2 homologous and closely linked loci, the HSD3B1 and HSD3B2 genes, which are located in chromosome band 1p13 (Appendix 1).[28,29] The type II enzyme encoded by the HSD3B2 gene is expressed in androgenic tissues,[28] and therefore, may regulate DHT levels by initiating inactivation of this potent androgen in the prostate.

A complex $(TG)_n$ $(TA)_n$ $(CA)_n$ repeat[30] in the HSD3B2 gene has been reported to be polymorphic, consisting of at least 25 different alleles.[31] Racial/ethnic diversity of this complex repeat in the HSD3B2 locus parallels prostate cancer risk,[31] suggesting that this locus may also have a role in prostate cancer predisposition and/or progression. The HSD3B2 gene encodes a bifunctional enzyme with dehydrogenase and isomerase activity.[28] Biochemical analysis of natural and in vitro generated mutants may lead to functional discrimination of these 2 activities.

OTHER ANDROGEN-METABOLIC CANDIDATE GENES

Other candidate genes can be probed using the same molecular epidemiological criteria used for the AR, SRD5A2 and HSD3B2 genes. For example, the enzyme cytochrome p450c17 (or steroid 17 α-hydroxylase/17,20 lyase) is the product of the CYP17 gene, and catalyzes 2 sequential reactions in the biosynthesis of testosterone in the gonads and the adrenal (Appendix 1).[9] A polymorphic T to C transition (A1/A2 allele) has been identified in the 5′ untranslated region of the CYP17 gene.[32,33] This polymorphism has been reported at increased frequency in white men with prostate cancer compared with controls.[32,33] However, a more recent study reported no significant difference in the frequency of this SNP between white patients and controls except for patients with a family history of the disease.[34] Another androgen-metabolic gene that may have a role in prostate cancer predisposition is the HSD17B3 locus, which encodes the testicular 17β-hydroxysteroid dehydrogenase type III that reduces androstenedione to testosterone.[9,35] No HSD17B3 polymorphisms have yet been reported in prostate cancer.

CONCLUSIONS

We presented the evidence and rationale supporting the involvement of a series of androgen-metabolic candidate genes in prostate cancer. Selection of these loci was guided by endocrinological, epidemiological, biochemical and molecular criteria (Appendixes 1 and 2).

This strategy uses polymorphic markers as the initial tool to analyze the natural genetic variation in candidate genes for multifactorial phenotypes such as prostate cancer (Appendixes 1 and 2). This molecular epidemiological approach has general applicability. SNPs[17] are identified and their individual contributions to the phenotype are characterized in concert with other allelic variants in the same or other genes and eventually in conjunction with environmental exposure. Molecular epidemiological investigations are supported by appropriate biochemical and pharmacological studies (in vitro and/or in vivo) that determine the functional significance of each allelic variant.[18,19] These multidisciplinary investigations are likely to involve endocrinological, epidemiological, pharmacological, molecular and biochemical methods. This convergence of various disciplines is likely to yield an integrated molecular view of complex disease phenotypes (Appendix 2), which will be complemented by analysis of environmental contributions.

Molecular epidemiological analyses of androgen-metabolic

genes in prostate cancer have proven fruitful for extending our knowledge of the predisposition to and progression of this disease. Further investigations are likely to yield additional advances in understanding this significant public health problem. Extensions of this approach to other androgen dependent diseases, such as benign prostatic hyperplasia, are also likely to lead to important insights into the etiology of these human diseases.[36]

The approach presented focused exclusively on sporadic prostate cancer. However, there are 2 forms of the disease, a common, sporadic form and a much more infrequent familial form, a dichotomy typical of many complex human phenotypes.[37] It is noteworthy in this context that none of the genes discussed in this review maps to any of the regions that are associated by linkage with the familial disease. This finding suggests that the more common, sporadic form of prostate cancer and the infrequent familial form may have distinct etiologies. This hypothesis may also have significant implications for the investigation of other complex human phenotypes.

Finally, we note that SNP discovery is an important goal of the Human Genome Project and SNPs are likely to come into widespread use in the near future.[17] Unfortunately, little attention is currently being paid to the examination of the functional significance of SNPs, perhaps because of the variability of the assays required to analyze the function of each SNP. The molecular data we reviewed have implications for association and linkage studies with SNP markers. Specifically, the epidemiological and kinetic data on the A49T missense mutation in the SRD5A2 gene highlight the enormous importance of functional studies for all SNP investigations. Therefore, rigorous functional analyses should be pursued for all SNPs throughout the genome, however time-consuming this may be.

We propose that SNPs be classified as functionally neutral or functionally significant based on appropriate experimental data (Appendix 3). The former may be best used in linkage studies, while the latter become "candidate alleles" (or "candidate SNPs") in candidate genes. In other words, functionally neutral SNPs are useful in identifying appropriate candidate genes for each phenotype, while functionally significant SNPs are useful for epidemiological (ie association) and pharmacological studies because they are more likely to actually cause the phenotype. Therefore, the molecular epidemiological analysis of prostate cancer may have resulted in the discovery of significant new strategies for human molecular genetic and epidemiological research in multifactorial phenotypes.

APPENDIX 1. CHARACTERISTICS OF ANDROGEN-METABOLIC GENES STUDIED IN PROSTATE CANCER

Gene	Gene Product	Chromosomal Location	Constitutional Mutations	Somatic Mutations
AR	Androgen receptor	Xq11–12	References 11, 13, 14, 15	References 13, 14, 15
SRD5A2	Steroid 5α-reductase	2p23	References 17, 24, 25	Reference 27
HSD3B2	3β-Hydroxysteroid dehydrogenase	1p13	Reference 31	None reported
CYP17	Cytochrome p450c17	10q24.3	References 32, 33	None reported

The presence of constitutional and/or somatic mutations refers to DNA from either lymphocytes or affected tumor tissue, and only polymorphic variants[31] have been reported in the HSD3B2 gene.

APPENDIX 2. SELECTION CRITERIA FOR CANDIDATE GENES IN MULITFACTORIAL PHENOTYPES, SUCH AS PROSTATE CANCER

- The gene product is in a pathway that has been shown to be involved in the etiology of the disease (eg androgen metabolism in the case of prostate cancer).
- The gene is expressed in the appropriate tissue.
- Polymorphic markers exist in the gene that can be used in association studies.

APPENDIX 3. IDENTIFICATION AND CHARACTERIZATION OF CANDIDATE SNPS FOR ASSOCIATION STUDIES IN MULTIFACTORIAL PHENOTYPES, SUCH AS PROSTATE CANCER

- The gene has been shown to be involved in the phenotype of interest.
- The gene is sequenced in many individuals (patients and controls from different racial/ethnic groups) to identify all SNPs.
- SNPs are characterized functionally.
- "Candidate SNPs" (or "candidate alleles") are selected based on functional data.
- Epidemiological studies investigate the role of "candidate SNPs" in the phenotype of interest.

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DISCUSSION

Dr. Eric Klein. Do you have any sense of the population frequency of the alleles that actually have functional consequences? Considering the complexity of the androgen pathway, it seems statistically unlikely that many individuals will have SNPs that all lead to much higher levels of DHT or lead to much lower levels. It is more likely that a person will have some SNPs that increase the reaction rate and some that decrease it. How is that all going to play out?

Dr. Juergen K. Reichardt. In the type 2 5α-reductase, which is where we have most of the data, the allele frequency of V89L is in excess of 40% in Asians and about 20% in black men. A49T, which was the variant that increased enzyme activity, has a frequency that varies slightly from population to population but it is in the 3% to 5% range. Therefore V89L and A49T are not that rare. Next comes the question of how is this going to play out intragenically and intergenically. You can have multiple SNPs on a single gene, and the other hypothesis is that you will have combinations of SNPs. If we can build high or low risk genotypes, even for a fraction of the population, it will be helpful for a variety of uses, such as in pre-symptomatic diagnosis for treatment. Clearly this will not be the one and only answer to prostate cancer, but it probably will explain a fraction of prostate cancers.

Dr. Elizabeth A. Platz. Were these transfections in the enzymatic activity studies?

Doctor Reichardt. First, we obtain cDNA by mutagenesis and then we put in the SNPs that we want, including multiple SNPs, if necessary. Next, we transfect the cDNA into cells that do not have an endogenous enzyme background and we measure the activity. We actually are measuring apparent constants, apparent K_M and apparent K_i. In the long run the enzyme and enzyme variants need to be purified to determine the biochemical and structural bases for some of these variations in activity.

Doctor Platz. Have you looked at all of the common haplotypes and all of the possible combinations to see how the variety of changes influences enzyme activity? For example, if you had V89L and A49T SNPs on the same chromosome, how would that affect activity?

Doctor Reichardt. In all the combinations we have done so far, V89L is a partial dominant, suppressing the activity of other variants on the gene. If the variants are on 2 different chromosomes, you actually get the average of the 2 activities, which may suggest that this is probably a monomeric enzyme and not an oligomeric enzyme.

Doctor Platz. Findings from the few studies that have looked at polymorphisms and 5α-reductase type 2 in relation to prostate cancer are not fully consistent. I was wondering if it depends on the haplotype frequencies in any particular population, and we have just not looked at it in that way yet.

Doctor Reichardt. There are a variety of issues involved, one being that looking at single SNPs may not be the complete answer. I think there are also differences in populations. I agree with you entirely that these findings have not always been replicated. I think that this is likely to be a continuing problem as long as we look at single SNPs.

Dr. Ian M. Thompson. Is there any way to quantitate and integrate all of these variants and to look for the androgen stimulus, enabling you to say that 1 variant will take you up and another will take you down, as opposed to a wild-type?

Doctor Platz. You could attempt to model it statistically. In fact, you can use haplotype analysis not only for SNPs in 1 gene, but also across genes. Another approach would be like path analysis, which to me is still kind of questionable. A third possibility would be total androgenic stimulation *in vitro*.

Doctor Reichardt. I think the more realistic approach for the next few years will be to try and construct high risk and low risk genotypes, taking a variety of genes into account, including HSD3B2, HSD17B3, 5α-reductase and the androgen receptor among others. This is probably going to be an epidemiological analysis in a huge population. The other approach, which is a bit more in the future, would be to try to reconstruct the entire pathway in a cell based system, where you then put in the different variants and try to model androgenic stimulation. I think technically that this approach is going to be a lot more difficult to do.

Doctor Thompson. How many variants are you talking about in the 5A2 pathway you are describing?

Doctor Reichardt. There are 10 in the 5A2 pathway and probably more in the A1. Of course, some are so rare that they are not of interest. HSD17B3 is involved in biosynthesis, and HSD3B2 is involved in biosynthesis and degradation. You would want to focus on those SNPs and those combinations that occur reasonably commonly. This is why epidemiologists are going to have to lead the way. In laboratory experiments, in addition to the difficulty of putting all of those genes in and expressing them coordinately, you cannot put in all of the variants. The in vitro work is relatively labor intensive. These assays were actually developed in the 1950s and they are slow.

Doctor Thompson. Can you speculate on how you would put together a project to look at this in the Prostate Cancer Prevention Trial (PCPT), other than conducting a retrospective analysis with a segment of PCPT to see if there is a difference?

Doctor Reichardt. If you are thinking solely about 5α-reductase inhibition, ideally you would develop a drug that is essentially insensitive to genotype. Finasteride has a 60-fold pharmacogenetic variation when looked at *in vitro*, which is significant. Some of this may have to come from rational drug design. When I talk about purifying the enzyme and doing some structural work, it would not be just for biochemical interest, but it could lead to rational drug design. If we knew what the different variants do biochemically, we could design a drug in which it does not matter if you change 1 amino acid for another, and the drug would work in everybody.

Dr. Neil Fleshner. Is it possible that the whole thing is an artifact of detection, meaning that if you have the allele it is harder to find your cancer? Or you may have a bias based on a biopsy, and it is the same bias you are trying to study.

Doctor Reichardt. We found that A49T occurs in controls and that prostate specific antigen (PSA) levels are increased in men with the A49T variant who are controls. In fact, it is a dose-response effect, so that heterozygotes have slightly increased levels and homozygotes have much more increased levels.

Doctor Fleshner. There was concern in the finasteride trial that patients might have a bias towards more cancer because they had smaller transition zones and larger peripheral zones at biopsy. If you have 1 of these alleles, it is like taking finasteride. Maybe these people just have a higher chance of having variants detected, making them a risk factor for detection as opposed to a biological risk.

Doctor Thompson. I wonder what testosterone and DHT do to the androgen response elements in PSA. These SNPs are all in 5α-reductase, type 2, meaning you vary testosterone and DHT concentrations within the prostate. What happens to PSA production under those different environments?

Doctor Fleshner. Finasteride lowers PSA.

Doctor Reichardt. A49T should be doing the opposite, that is reducing testosterone and increasing DHT. We have a plausible biological rationale for what the SNP should be doing, which is to change androgen metabolism, but we do not have any direct evidence.

Doctor Thompson. PCPT will answer that question unless it is a biopsy bias. Men with a low PSA and normal rectal examination are controls, while those with an increased PSA, abnormal digital rectal examination and positive biopsy become patients cases.

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Advances in Brief

Breast Cancer Susceptibility Gene 1 (*BRCA1*) Is a Coactivator of the Androgen Receptor[1]

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Abstract

In the present study, the role of BRCA1 in ligand-dependent androgen receptor (AR) signaling was assessed. In transfected prostate and breast cancer cell lines, BRCA1 enhanced AR-dependent transactivation of a probasin-derived reporter gene. The effects of BRCA1 were mediated through the NH_2-terminal activation function (AF-1) of the receptor. Cotransfection of p160 coactivators markedly potentiated BRCA1-mediated enhancement of AR signaling. In addition, BRCA1 was shown to interact physically with both the AR and the p160 coactivator, glucocorticoid receptor interacting protein 1. These findings suggest that BRCA1 may directly modulate AR signaling and, therefore, may have implications regarding the proliferation of normal and malignant androgen-regulated tissues.

Introduction

Women who inherit loss-of-function germ-line mutations in *BRCA1*[5] have an increased lifetime risk of developing breast and/or ovarian tumors (1). The *BRCA1* gene product is a nuclear phosphoprotein with putative roles in DNA repair, cell cycle control, and transcriptional regulation (2). There is some evidence to suggest that wild-type BRCA1 functions indirectly or directly in the regulation of endocrine signaling pathways: (*a*) although every cell in an affected individual possesses the same germ-line *BRCA1* mutation, tumors arise exclusively in the breast and ovary, two hormone-regulated tissues; (*b*) wild-type BRCA1 inhibits estrogen receptor α signaling in transfected breast and prostate cancer cell lines (3); and (*c*) breast cancer penetrance among *BRCA1* mutation carriers is modified by allele variation at the *AR* locus (4). Because of the importance of AR signaling in the regulation of prostate and mammary epithelial cell proliferation, we investigated the potential role of BRCA1 in ligand-dependent AR transactivation. Herein, we show that BRCA1 enhances AR signaling in both prostate and breast cancer cell lines, especially in the presence of exogenous p160 coactivator. We further present *in vitro* evidence that BRCA1 makes direct contacts with the AR and with the p160 coactivator, GRIP1.

Materials and Methods

Plasmids. Mammalian expression or reporter plasmids pCMV-hAR (5), pSG5-GRIP1 and pSG5-SRC-1a (6), pcDNA3.1-AIB1 (7), ARR_3tk-CAT (8), and pcDNA-hAR(Q)$_n$ (9) were described previously. To construct vector pcDNA-AR (NTD-DBD), an *Nhe*I-*Bam*HI fragment was PCR amplified from pcDNA-hAR (9) plasmid DNA using primer pairs S1 (5′-GTGGGCAGCTAGCTGCAGCGACTAC-3′) and AS1 (5′-ATGGAGGGATCCTCAGGTGCTGGAAGCCTCTCCTTC-3′) and inserted into the reciprocal restriction sites of pcDNA3.1(+) (Invitrogen, Carlsbad, CA). Vector pcDNA-AR (DBD-LBD) was constructed in sequential cloning steps: (*a*) an *Nhe*I-*Kpn*I PCR fragment containing the AR Kozak sequence was amplified using primers S1 and AS2 (5′-ACCTAAGGTACCCCCTAACTGCACTTCCATCCT-3′) and inserted into the corresponding sites of pcDNA3.1(+); (*b*) a *Kpn*I-*Eco*RI PCR fragment was amplified using primers S2 (5′-AATCGCGGTACCCGTTTGGAGACTGCCAGGGACCAT-3′) and AS3 (5′-GGAAATGAATTCG-GGGAAATAGGGTTTCCAAT-3′) and inserted into the restored *Kpn*I site and the downstream *Eco*RI site of the pcDNA3.1(+) multiple cloning site. BRCA1 mammalian expression vector pcDNA-BRCA1 was constructed by inserting a *Not*I-*Xho*I treated BRCA1 fragment derived from pBSK-1hFL plasmid (10) into the corresponding restriction sites of pcDNA3.1/mycHisC(-) vector (Invitrogen). Bacterial expression plasmids encoding GST, GST-AR, and GST-GRIP1 fragments were described previously (6, 11).

Tissue Culture and Transfections. Cells obtained from the American Type Culture Collection (Manassas, VA) were maintained in RPMI (PC-3, DU-145, and HBL-100 cells) or DMEM (MCF-7 cells) medium that contained 10% FBS. Approximately 24 h prior to transfection, 10^6 (PC-3, DU-145, and HBL-100) or 5×10^5 (MCF-7) cells were seeded into each 60-mm dish. Cells were transfected in serum-free conditions with Lipofectamine reagent (Life Technologies, Inc.) according to the manufacturer's protocol. In each experiment, the total amount of DNA per dish was held constant by the addition of pcDNA3.1(+) vector when appropriate. After transfection, cells were grown for 24 h (DU-145, HBL-100, and MCF-7) or 48 h (PC-3) in RPMI 1640 (without phenol red) that contained 5% charcoal/dextran-stripped FBS (Gemini Bio Products, Calabasas, CA) and, where indicated, DHT (1 or 10 nM) for the last 24 h of growth. Whole-cell extracts were prepared in 0.25 M Tris-HCl (pH 8.0) by repeated freezing and thawing. CAT assays were performed using the Quan-T-CAT kit (Amersham Pharmacia Biotech, Piscataway, NJ), and total cellular protein was measured using the Bio-Rad (Hercules, CA) Protein Assay kit. Relative CAT activities (cpm/A_{600}) are reported as the mean + SE of three independent dishes.

GST Pull-Downs. GST and GST-fusion proteins were expressed and purified as described previously (12). Glutathione-Sepharose-bound GST protein, GST-AR (1–555), or GST-GRIP1 fragments (5–765, 563-1121, or 1121–1462) were incubated with ^{35}S-radiolabeled full-length BRCA1 transcribed and translated *in vitro* from pcDNA3.1 vectors. Associated BRCA1 was eluted,

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[2] These authors contributed equally to this work.

[3] The contributions of the Press and Coetzee laboratories were equal.

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[5] The abbreviations used are: BRCA1, breast cancer susceptibility gene 1; AIB1, amplified in breast cancer-1; AF, activation function; AR, androgen receptor; CAT, chloramphenicol acetyltransferase; DBD, DNA binding domain; DHT, dihydrotestosterone; FBS, fetal bovine serum; GRIP1, glucocorticoid receptor interacting protein 1; GST, glutathione *S*-transferase; LBD, ligand binding domain; NLS, nuclear localization signal; NR, nuclear receptor; NTD, NH_2-terminal domain; SRC-1a, steroid receptor coactivator 1a.

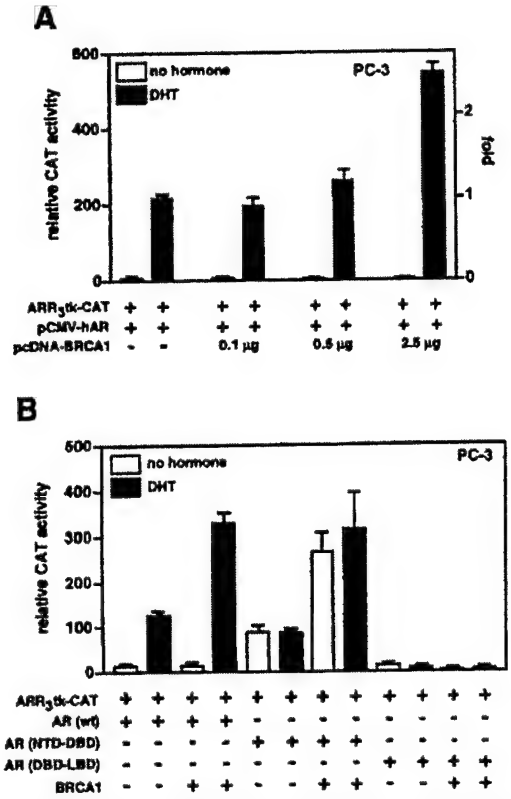

Fig. 1. BRCA1 potentiates AR signaling. *A*, wild-type BRCA1 coactivates AR transactivation in PC-3 prostate cancer cells. Transiently transfected cells were assayed for stimulation of ARR_3tk-CAT reporter activity by DHT. ARR_3tk-CAT is composed of a minimal thymidine kinase (*tk*) promoter under the control of three identical fragments of the rat probasin promoter (nucleotides −244 to −96), each comprising two androgen receptor binding sites (*i.e.*, ARBS-1 and ARBS-2; Ref. 8). Cells were cotransfected with 2.0 μg of ARR_3tk-CAT, 50 ng of pCMV-hAR, and increasing amounts of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; *bars*, SE. Fold is measured relative to DHT-dependent AR activity with no transfected BRCA1. *B*, BRCA1 works through AR AF-1. PC-3 cells were cotransfected with 50 ng of pCMV-hAR, 10 ng of pcDNA-AR (NTD-DBD), or 0.5 μg of pcDNA-AR (DBD-LBD), 2.0 μg of ARR_3tk-CAT, and 2.5 μg of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; *bars*, SE. Mammalian expression vectors pcDNA-AR (NTD-DBD) and pcDNA-AR (DBD-LBD) encode AR amino acids 1–647 and 538–919, respectively. AR (NTD-DBD) is a constitutive activator of ARR_3tk-CAT, and thus, potentiation of its activity by BRCA1 is ligand independent.

resolved by SDS-PAGE, and analyzed by autoradiography. Ten % of the total labeled BRCA1 incubated in each reaction was loaded for comparison.

Western Analysis. Approximately 24 h prior to transfection, 5×10^5 PC-3 cells were seeded into each 16-mm well. Cells were transfected with the Superfect reagent (Qiagen, Valencia, CA) according to the manufacturer's protocol. After transfection, cells were grown for 48 h in RPMI 1640 (without phenol red) that contained 5% charcoal/dextran-stripped FBS and, where indicated, 10 nM DHT. Transfected cells were harvested in RIPA buffer that contained mammalian protease inhibitors. Total cellular protein was measured by the BCA Protein Quantification Assay (Pierce, Rockford, IL), and equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham-Pharmacia Biotech, Piscataway, NJ) and probed with rabbit polyclonal anti-AR antibody N20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml.

Results

BRCA1 Enhances AR Signaling. To assess the role of BRCA1 in AR signaling, prostatic carcinoma cells (*i.e.*, PC-3 cells) were cotransfected with AR and BRCA1 expression vectors, as well as with the ARR_3tk-CAT probasin reporter. A 2.5-fold DHT-dependent potentiation of AR transactivation activity was observed with 2.5 μg of transfected pcDNA-BRCA1 vector (Fig. 1*A*). BRCA1 had no effect on AR signaling in the absence of DHT (Fig. 1*A*), and it failed to activate the reporter gene in the absence of exogenous AR (data not shown).

BRCA1 Works through AR AF-1. The AR, like all NRs, comprises three structural/functional domains: a poorly conserved NTD; a highly conserved DBD; and a COOH-terminal LBD (13). Both the NTD and LBD contain activation functions (*i.e.*, AF-1 and AF-2, respectively) that mediate the transcriptional activation potential of the receptor. To determine which AF primarily is involved in BRCA1-mediated coactivation of the AR, AR constructs containing either AF-1 (NTD-DBD) or AF-2 (DBD-LBD) were coexpressed in PC-3 cells with BRCA1. BRCA1 enhanced the constitutive transactivation activity of AR (NTD-DBD) nearly 3-fold, but failed to enhance AR (DBD-LBD) activity in either the presence or absence of DHT (Fig. 1*B*). Thus, BRCA1 can potentiate AR signaling through functional interactions with AR AF-1. It is important to note that BRCA1-dependent potentiation of AR signaling is not attributable to increased AR protein expression in the presence of coexpressed BRCA1 (Fig. 2).

BRCA1 and the p160 Coactivators Synergistically Potentiate AR Signaling in Prostate and Breast Cancer Cell Lines. The p160 coactivators are a family of M_r 160,000 nuclear proteins that bind to NRs and potentiate ligand-dependent receptor signaling by recruiting to the target promoter a large, multisubunit coactivator complex that possesses histone acetyltransferase activity (14). The p160 coactivators interact with and coactivate the AR through both of its AFs (6). To determine whether BRCA1 plays a role in p160-mediated coactivation of the AR, PC-3 cells were cotransfected with expression vectors for the AR, BRCA1, and/or the p160 coactivators GRIP1, SRC-1a, and AIB1. As expected, BRCA1 and GRIP1 individually enhanced AR transactivation of the ARR_3tk-CAT reporter about 2- and 3-fold, respectively (Fig. 3*A*). When coexpressed, however, AR transactivation activity was enhanced 12-fold. This combined BRCA1-GRIP1 coactivation of AR signaling was synergistic because it was greater than the additive effects of BRCA1 and GRIP1 measured independently. Similar results were obtained when either SRC-1a or AIB1 were used, suggesting a generic BRCA1-p160 functional interaction (Fig. 3*A*).

To rule out the possibility that the observed BRCA1 effects on AR signaling were specific to PC-3 cells, an additional prostate carcinoma cell line (*i.e.*, DU-145), an SV40-transformed breast epithelial cell line (*i.e.*, HBL-100), and a breast cancer cell line (*i.e.*, MCF-7), were used in cotransfection experiments (Fig. 3*B*). In DU-145 cells, as in PC-3 cells, BRCA1 and GRIP1 individually enhanced DHT-depend-

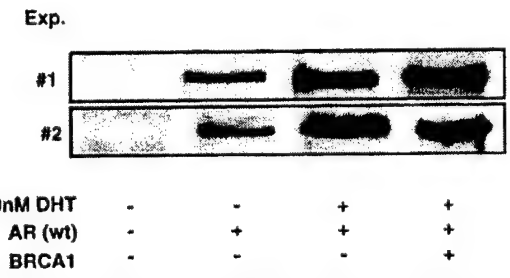

Fig. 2. AR protein expression is not stabilized by BRCA1 in transfected cells. PC-3 cells were cotransfected with 50 ng of pCMV-AR and 250 ng of pcDNA-BRCA1 as indicated. Forty-eight h after transfection, whole-cell extracts were prepared, and equivalent amounts of each extract were probed with anti-AR antibody. Autoradiograms from two independent experiments are presented. Radiographic bands were analyzed by scanning densitometry using a Bio-Rad Model GS-710 imaging densitometer. No significant increase in AR band density was observed in the presence of BRCA1 (data not shown).

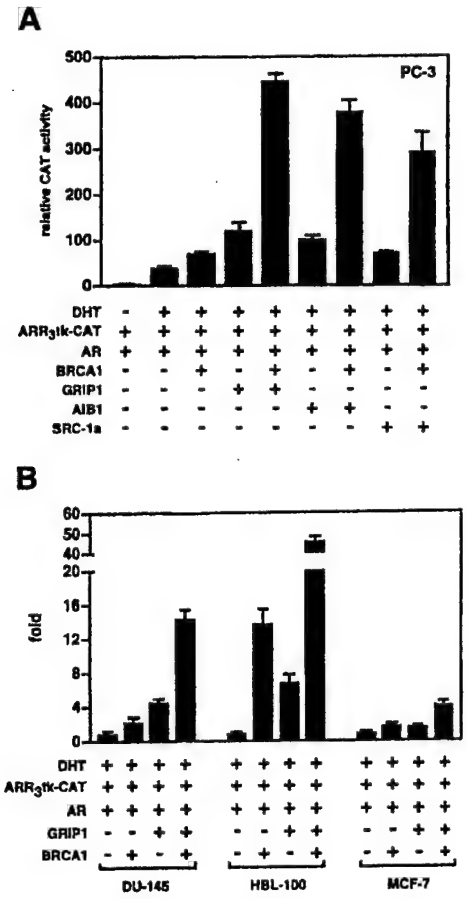

Fig. 3. Coactivation of AR signaling by BRCA1 and members of the p160 family of nuclear receptor coactivators is synergistic. *A*, PC-3 cells were cotransfected with 2.0 μg of pSG5-GRIP1, pcDNA3.1-AIB1, or pSG5-SRC-1a, 2.0 μg of ARR_3tk-CAT, 25 ng of pCMV-hAR, and 2.5 μg of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and data presented are the means of three independent dishes; *bars*, SE. *B*, potentiation of AR signaling by BRCA1 occurs in both prostate and breast cancer cell lines. Prostate cell line DU-145 and breast cell lines HBL-100 and MCF-7 were cotransfected with 2.0 μg of ARR_3tk-CAT, 25 ng of pCMV-hAR, 2.0 μg of pSG5-GRIP1, and 2.5 μg of pcDNA-BRCA1 as indicated. CAT activities were normalized to total cellular protein, and the data presented are fold relative to DHT-dependent AR activity with no transfected BRCA1 or GRIP1; *bars*, SE.

ent AR transactivation of the ARR_3tk-CAT reporter (*i.e.*, 2.5- and 5-fold, respectively). Likewise, when BRCA1 and GRIP1 were coexpressed in this cell line, a 14-fold synergistic coactivation of AR signaling was observed. In HBL-100 cells, the effects of BRCA1 were more dramatic. For example, BRCA1 alone potentiated AR transactivation activity greater than 12-fold. In combination with GRIP1, moreover, BRCA1 resulted in a nearly 45-fold enhancement of AR signaling. In MCF-7 cells, BRCA1 and GRIP1 individually potentiated AR activity <2-fold. Together, however, they did result in a 5-fold coactivation of AR signaling, consistent with observations made in the other cell lines. The relatively small BRCA1 effects seen in MCF-7 cells may be attributable to high endogenous p160 coactivator levels (7).

BRCA1 Interacts with the AR NTD and the GRIP1 COOH-Terminus. To determine whether BRCA1 makes physical contacts with the AR and/or GRIP1, GST pull-down experiments were performed in which *in vitro* translated and ^{35}S-labeled BRCA1 was incubated with immobilized GST-AR (amino acids 1–555) or with various GST-fused fragments of GRIP1 (*i.e.*, amino acids 5–765, 563-1121, or 1122–1462). In these experiments, BRCA1 interacted with GST-AR (1–555) and with GST-GRIP1 (1122–1462) but not with the other GRIP1 fragments (Fig. 4*B*).

Overexpression of BRCA1 Alleviates the Inhibitory Polyglutamine (Poly-Q) Effect on AR Transactivation Activity. We have shown previously that AR transactivation activity decreases with increasing poly-Q length and that this may be attributable to inhibition of p160-mediated coactivation (9). To assess the impact of AR poly-Q length on BRCA1 and BRCA1/GRIP1 coactivation of AR signaling, ARs with varying poly-Q lengths were expressed in PC-3 cells along with BRCA1 alone or in combination with GRIP1 (Fig. 5). As expected, DHT-dependent AR transactivation of the ARR_3tk-CAT reporter decreased modestly with increasing poly-Q length in PC-3 cells (Fig. 5, *white histograms*). This effect was not, however, observed when BRCA1 was coexpressed (Fig. 5, *gray histograms*). In addition, no inhibition of GRIP1 coactivation with increasing AR poly-Q length was observed in the presence of coexpressed BRCA1 (Fig. 5, *black histograms*).

Discussion

The data presented in this study support a direct role for BRCA1 in AR signaling. In both prostate and breast epithelial cell lines, BRCA1 enhanced ligand-dependent AR transactivation of an androgen-responsive probasin reporter gene. BRCA1, moreover, synergized

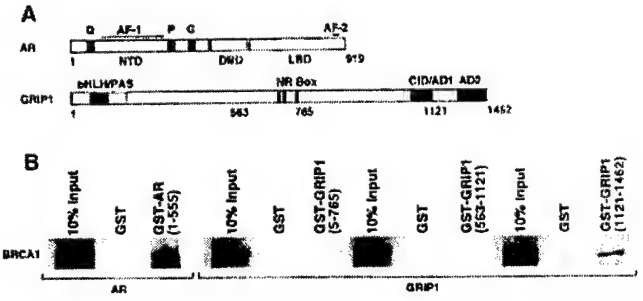

Fig. 4. BRCA1 interacts with the AR and GRIP1. *A*, schematic diagrams of the AR and GRIP1 showing the locations of various functional domains. Domains of AR: *AF-1/AF-2*, autonomous activation functions 1 and 2; *NTD*; *DBD*; *LBD*; *Q/P/G*, glutamine/proline/glycine poly-amino acid stretches. Domains of GRIP1: *bHLH*, basic helix-loop-helix sequence; *PAS*, Per-Arn-Sim domain; NR boxes, nuclear receptor binding domains (LXXLL motifs); *CID*, CBP interaction domain; *AD1/AD2*, activation domains. Numbers represent relative amino acid positions. *B*, full-length BRCA1 binds to the NH2-terminal domain of the AR and the COOH terminus of GRIP1. Unpurified *in vitro* translated BRCA1 was incubated with GST, GST-AR (1–555), GST-GRIP1 (5–765), GST-GRIP1 (563–1121) or GST-GRIP1 (1121–1462).

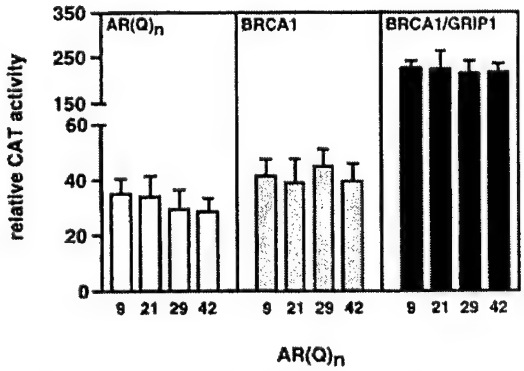

Fig. 5. BRCA1 normalizes the AR poly-Q effect. PC-3 cells were cotransfected with 2.0 μg of ARR_3tk-CAT, 25 ng of pcDNA-hAR(Q)$_n$, 2.0 μg of pSG5-GRIP1, and 2.0 μg of pcDNA-BRCA1 as indicated. CAT activities in the presence of DHT were normalized to total cellular protein and to AR(Q)$_n$ expression levels as determined by ligand binding assays (9). Data presented are the means of three independent dishes; *bars*, SE.

with the p160 coactivators to potentiate AR activity. In addition, BRCA1 physically interacted with both the AR and GRIP1, perhaps indicative of the formation *in vivo* of an AR/p160/BRCA1 ternary complex mediated by reciprocal interactions between the AR NTD, the GRIP1 COOH terminus, and BRCA1. In light of this, it is possible that BRCA1 participates in the formation and/or stabilization of the NR coactivator complex. Our data suggest that BRCA1 is directly recruited to androgen-responsive promoters through interactions with the AR NTD or with the GRIP1 COOH terminus. Once localized to the target promoter, BRCA1 may facilitate activated transcription by "bridging" communications between the bound NR coactivator complex and the RNA polymerase II-containing preinitiation complex, which associates with the BRCA1 COOH terminus (15). In this view, BRCA1 plays a critical role in modulating the effects of androgen signals on cells by increasing the efficacy and accuracy of AR-mediated transcriptional events. Loss of cellular BRCA1 function, therefore, perhaps because of mutations causing COOH-terminally truncated forms of the protein, would be expected to reduce the potency of AR-dependent signaling.

Several lines of evidence indicate that androgen signaling in the breast might in fact protect against cancer development and progression: (*a*) androgens have been used successfully to treat metastatic female breast cancers with comparable efficacy to tamoxifen, but the treatment was not well tolerated because of its masculinizing side effects (16); (*b*) androgens have been shown to inhibit the proliferation of AR-positive breast cancer cell lines in culture (17); (*c*) reduced or impaired AR signaling has been implicated in the development of hereditary male breast cancers (18); and (*d*) Rebbeck *et al.* (4) have reported an association between the polymorphic AR CAG repeat and breast cancer penetrance among *BRCA1* mutation carriers. In their study, women who carried at least one long AR CAG allele (*i.e.*, >27 repeats) had a significantly earlier age at diagnosis than women with only short alleles. Interestingly, breast cancer penetrance increased with increasing AR CAG length. Because of the well-characterized negative effect of increasing poly-Q length (encoded by the CAG repeat) on AR transactivation activity (9), it is tempting to speculate that reduced AR signaling encourages neoplastic transformation in mammary epithelial cells harboring *BRCA1* mutations. Our findings may indirectly support this idea because BRCA1 overexpression apparently abolishes the inhibitory effect of increasing poly-Q length on p160-mediated coactivation of the AR (Ref. 9; Fig. 5). It may be that in women with germ-line BRCA1 mutations (and therefore, with reduced functional BRCA1 protein), breast epithelial cells are under reduced androgen-mediated growth inhibition and tumors develop more rapidly in those women expressing less efficient ARs.

The results of this study, although still at an early stage, suggest a complex interplay between the AR, p160 coactivators, and BRCA1 that may be important in regulating epithelial cell proliferation and, by implication, cancer risk in certain hormone-regulated tissues like the breast and prostate. In the prostate, loss of BRCA1 function initially was associated with increased risk for cancer development (19), although more recent studies have failed to find specific *BRCA1* mutations at increased frequencies among prostate cancer patients (20). Decreased AR signaling because of loss of BRCA1 function might even protect against prostate cancer development and/or progression because most early-stage prostate cancers are androgen dependent. Nevertheless, it is difficult to reconcile the tumor suppressor functions of BRCA1 (*i.e.*, DNA repair and cell cycle control) with such a proposal. Clearly, future studies are needed to explore these important issues.

Acknowledgments

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Genetic Determinants of Serum Prostate-specific Antigen levels in Healthy Men from a Multiethnic Cohort[1]

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Abstract

We recently reported an association between prostate cancer risk and polymorphisms in the prostate-specific antigen (*PSA*) and androgen receptor (*AR*) genes. The purpose of this study is to test whether these two polymorphisms, *AR CAG* and *PSA ARE1*, influence serum PSA levels in healthy men. Serum PSA and the two genotypes were assayed for 420 healthy men from a multiethnic cohort, and regression models were fit to estimate the effects of *AR CAG* genotype and *PSA ARE1* genotype on serum PSA levels. Predicted serum PSA decreased 3.5% with each additional *AR CAG* repeat decile ($P = 0.01$). Serum PSA was also associated with *PSA ARE1* genotype, with PSA levels higher among men with the *PSA AA* genotype compared with men with the *AG* or *GG* genotypes ($P = 0.02$). The relationship between serum PSA level and *AR CAG* length differed according to *PSA* genotype ($P = 0.049$): for genotype *GG*, the slope was not significantly different from zero ($P = 0.74$); for genotype *AG*, serum PSA increased 4.5% with each decrease of one *CAG* repeat decile ($P = 0.03$); for genotype *AA*, serum PSA increased 7% with each decrease of one *CAG* repeat decile ($P = 0.02$). These results indicate that in healthy men, genetic variants in the *PSA* and *AR* genes contribute to variation in serum PSA levels. Men with the *PSA AA* genotype and short *AR CAG* alleles have, on average, higher serum PSA levels.

Introduction

Serum PSA[3] is widely used as a tumor marker for early detection of prostate cancer. PSA, however, is not cancer specific. Benign prostatic epithelial cells also produce PSA. Any condition that increases prostate size, such as benign prostatic hyperplasia, or that disrupts the prostatic architecture, such as prostatitis, prostatic ischemia, or infarction, can elevate serum PSA. Serum PSA gradually increases with age, because of a progressive increase in prostate size (1). Racial differences in serum PSA levels have been noted, with African-American men having markedly higher serum PSA levels than their Caucasian counterparts (2, 3), perhaps attributable in part to larger average prostate volume. Other factors that might influence PSA levels include those factors that directly or indirectly regulate *PSA* gene expression.

The major regulator of *PSA* gene expression is androgen. The AR, after binding to ligand (androgen), recognizes and binds to specific nucleotide sequences, called AREs, in the promoter regions of androgen-regulated genes. At least three AREs have been identified in the *PSA* gene promoter (4). The one nearest the transcription start site is referred to as *ARE1*. We recently reported that a single-nucleotide polymorphism in the ARE1 sequence was associated with prostate cancer risk and, furthermore, that this association may be modified by allelic variation in the *AR* gene (5). In this study, we set out to test whether polymorphisms in these two genes, *PSA* and *AR*, influence serum PSA levels in healthy men.

Materials and Methods

Subjects. Subjects were 456 men participating in the Hawaii-Los Angeles Multiethnic Cohort Study of diet and cancer. The male cohort consists of ~13,000 African Americans, 23,000 Hispanics, 27,000 Japanese Americans, and 23,000 non-Hispanic whites who were between the ages of 45 and 75 at entry into the cohort. All of the cohort members have completed a detailed health and dietary questionnaire and are periodically traced, primarily through population-based cancer registries, for occurrence of all incident cancers. Blood and urine specimens are collected from all incident cancer cases and from a 3% random sample of the cohort. *AR CAG* genotypes had been performed on approximately the first 1,000 samples (cases and controls) collected. Men eligible for the current study were those who have not been diagnosed with prostate cancer and for whom *AR CAG* genotypes were already available. Because our aim was to study men having normal prostate function, we excluded 36 men who had serum PSA levels above 4 ng/ml, leaving 420 men (100 African Americans, 113 non-Hispanic whites, 108 Hispanics, and 99 Japanese Americans) in the study. Forty men (9.5%) reported a history of prostate enlargement. Excluding these men did not alter our results. Written informed consent was obtained from each subject. The study was approved by the University of Southern California School of Medicine Institutional Review Board.

Genotyping. Two genes, *AR* and *PSA*, were examined in this study. In the *AR* gene, two microsatellite polymorphisms (*CAG* and *GGC*) in exon 1 were genotyped using methods described in our previous report (6). These microsatellites are length polymorphisms, with individual alleles defined by the number

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[3] The abbreviations used are: PSA, prostate-specific antigen; AR, androgen receptor; ARE, androgen response element; IGF, insulin-like growth factor.

Table 1 Age and serum PSA levels in study population by ethnicity

	African Americans	Non-Hispanic whites	Hispanics	Japanese Americans
n	100	113	108	99
Age				
Mean (SD)	62.2 (8.2)	62.2 (8.7)	64.1 (8.0)	62.5 (9.7)
Median (25th, 75th percentile)	62 (57, 68)	62 (55, 69)	65 (58, 72)	62 (54, 71)
PSA				
Mean (SD)	1.3 (0.9)	1.2 (0.9)	1.3 (1.0)	1.2 (0.8)
Median (25th, 75th percentile)	1.0 (0.6, 1.7)	1.0 (0.5, 1.6)	1.0 (0.6, 1.7)	1.0 (0.6, 1.7)

Table 2 Distribution of *AR* and *PSA* genotypes by ethnicity

Genotype	African Americans (n = 100) n (%)	Non-Hispanic whites (n = 113) n (%)	Hispanics (n = 108) n (%)	Japanese Americans (n = 99) n (%)	All of the groups (n = 420) n (%)
AR CAG					
7–16	23 (23)	4 (4)	5 (5)	2 (2)	34 (8)
17–18	23 (23)	10 (9)	9 (8)	5 (5)	47 (11)
19	8 (8)	18 (16)	6 (6)	6 (6)	38 (9)
20	13 (13)	17 (15)	19 (18)	9 (9)	58 (14)
21	13 (13)	12 (11)	15 (14)	19 (19)	59 (14)
22	5 (5)	15 (13)	14 (13)	26 (26)	60 (14)
23	1 (1)	14 (12)	13 (12)	15 (15)	43 (10)
24	5 (5)	13 (12)	12 (11)	8 (8)	38 (9)
25–26	5 (5)	8 (7)	7 (6)	5 (5)	25 (6)
27–37	4 (4)	2 (2)	8 (7)	4 (4)	18 (4)
AR GGC[a]					
3–15	42 (45)	14 (12)	12 (11)	19 (19)	87 (21)
16	21 (23)	66 (59)	65 (61)	56 (57)	208 (51)
17–20	30 (32)	32 (29)	30 (28)	23 (24)	115 (28)
PSA ARE1					
AA	28 (28)	28 (25)	12 (11)	5 (5)	73 (17)
AG	48 (48)	52 (46)	56 (52)	31 (31)	187 (45)
GG	24 (24)	33 (29)	40 (37)	63 (64)	160 (38)
PSA −252/−203[b]					
GG/ΔAΔA	84 (87)	52 (49)	49 (47)	38 (40)	223 (56)
GA/AΔA	12 (13)	46 (43)	46 (44)	50 (52)	154 (38)
AA/AA	0 (0)	8 (8)	9 (9)	8 (8)	25 (6)

[a] *AR GGC* genotype missing for 10 samples.
[b] *PSA* −252/−203 genotypes missing for 18 samples.

of repeated units (*CAG* or *GGC* repeats) that they contain. Genotypes were assayed by separating radioactively labeled PCR products on polyacrylamide gels. *GGC* genotype was missing for 10 subjects because of PCR failure.

In the *PSA* gene promoter, a *G/A* substitution polymorphism in the ARE1 sequence was genotyped using methods described in our previous report (5). PCR products were digested with the *Nhe*I enzyme (New England Biolabs, Beverly MA) and genotypes were distinguished by running digested products on agarose gels: *AA* (300 bp), *AG* (150 and 300 bp), and *GG* (150 bp). Additionally, a 560-bp region surrounding this polymorphism was sequenced for all subjects using primers GTTGGGAGTGCAAGGAAAAG (forward) and GGACAGGGTGAGGAAGACAA (reverse). For 18 subjects, the complete sequence was not readable because of poor template quality.

Serum PSA Levels. Serum PSA levels were performed by the University of Southern California Norris Cancer Hospital Clinical Laboratory using a two-site immunoenzymometric assay with a Hybritech anti-PSA mouse monoclonal antibody (TOSOH Medics, Inc., Foster City, CA). The minimal detectable PSA concentration was 0.05 ng/ml, with intra-assay and interassay coefficient of variations of 2.9 and 2.1%, respectively.

Statistical Methods. Serum PSA levels were log transformed, and linear regression models were fitted to estimate the effects of *AR* and/or *PSA* genotypes on serum PSA levels, adjusting for age and ethnicity. Because a few observations with extremely long or extremely short *AR CAG* length were highly influential in determining regression coefficients, *CAG* length was grouped into approximate deciles to improve robustness of the models. Decile 1 corresponds to 7–16 *CAG* repeats, decile 2 to 17- 18 *CAG* repeats, each of deciles 3 through 8 corresponds to a single *CAG* repeat category (19–24 repeats, respectively), decile 9 corresponds to 25–26 *CAG* repeats, and decile 10 to 27–37 *CAG* repeats. The medians of the decile groups: 15, 17, 19, 20, 21, 22, 23, 24, 25, and 28 *CAG* repeats, were used as scores for coding *CAG* length in the regression equations. The resulting regression coefficient can be interpreted as representing the additive increase in ln(PSA), and the exponentiated coefficient as the multiplicative increase in PSA for each decrease of one *CAG* unit. Heterogeneity tests were performed by calculating the likelihood ratio statistic, comparing the model

Table 3 PSA promoter haplotype frequencies (%) by ethnicity

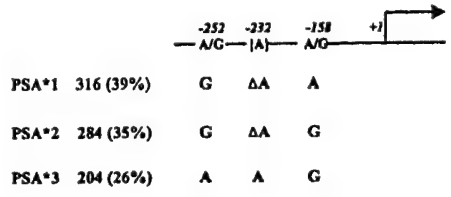
	African Americans (n = 96)	Non-Hispanic whites (n = 106)	Hispanics (n = 104)	Japanese Americans (n = 96)
*PSA*1*	53	46	37	20
*PSA*2*	41	25	32	45
*PSA*3*	6	29	31	35

Fig. 1. Correspondence between *PSA* genotypes and haplotypes. *A*, classification of 402 subjects according to *PSA* genotypes at positions −252, −232, and −158. *B*, classification of 804 chromosomes according to PSA haplotypes (*PSA*1*, *PSA*2*, and *PSA*3*).

A

-252	-232	-158 (ARE1) AA	AG	GG
GG	ΔA/ΔA	69 (17%) *PSA*1/*1*	104 (26%) *PSA*1/*2*	50 (12%) *PSA*2/*2*
GA	ΔA/A	0 (0%)	74 (18%) *PSA*1/*3*	80 (20%) *PSA*2/*3*
AA	A/A	0 (0%)	0 (0%)	25 (6%) *PSA*3/*3*

B



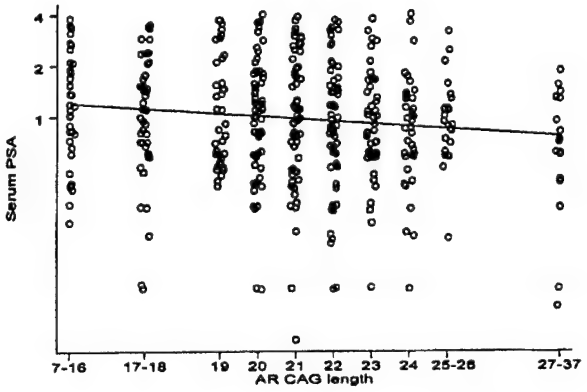


Fig. 2. Serum PSA as a function of *AR CAG* length. ○, a single subject. *Line*, regression of ln(PSA) on *AR CAG* length, adjusted for age and ethnicity.

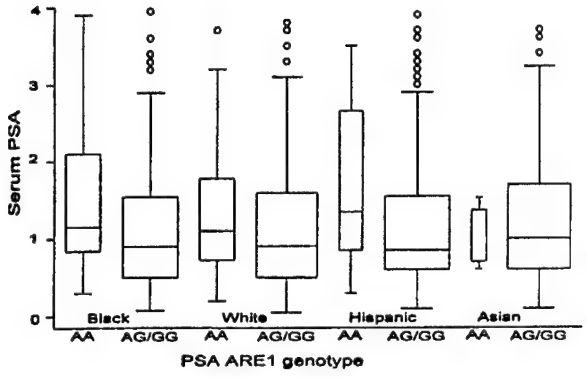


Fig. 3. Distribution of serum PSA levels by *PSA ARE1* genotype and ethnicity. The width of each box is proportional to the square root of the number of observations in the group.

with a single regression line to a model with separate regression lines for each genotypic group. All *P*s were two-sided.

Because all of the *AR GGC* genotypes other than genotype 16 were relatively uncommon, *GGC* alleles were categorized as <16, 16, and >16, roughly corresponding to the bottom quartile, middle 50%, and upper quartile, respectively. *GGC* genotype group was modeled by including two indicator variables in the regression model.

Results

The age of the subjects at the time of blood collection ranged from 47 to 80 years, with a mean of 62.8 years. Mean and median ages were slightly higher for Hispanics than for other ethnic groups, but these differences were not statistically significant (Table 1). There were no significant ethnic differences in serum PSA (Table 1), either before or after adjusting for age. Age was weakly correlated with serum PSA ($R = 0.16$; $P < 0.01$).

The distributions of *AR* and *PSA* allele frequencies are shown in Table 2. Ethnic-specific allele frequencies were similar to those previously reported (5, 6). In the *AR* gene, short *CAG* length (<19 *CAG* repeats) and short *GGC* length (<16 *GGC* repeats) were more common, and *GGC* length of 16 was less common among African Americans than among men of other ethnic groups. At the *PSA ARE1* locus, the *G* allele was most frequent among Japanese Americans, intermediate among Hispanics, and least frequent among African Americans and non-Hispanic whites. Genotype frequencies were in agreement with Hardy-Weinberg equilibrium in all of the ethnic groups (data not shown).

In the *PSA* gene promoter, two new polymorphisms were identified. An *A/G* polymorphism at −252, 79 bp upstream from *ARE1*, and a single-base deletion polymorphism (*A/ΔA*) at −232. The two polymorphisms were in perfect linkage disequilibrium, with the *−252A* allele always corresponding to the *−232A* allele and the *−252G* to the *−232ΔA* allele (see Fig. 1). The *−252A/−232A* allele was much less common among African Americans than among men of other ethnic groups; in fact no *−252A/−232A* homozygotes were observed among African Americans (Table 2). The two polymorphisms were also in linkage disequilibrium with the *ARE1* polymorphism, with the *−252A/−232A* allele occurring only in combination with the *ARE1G* allele (Fig. 1). Thus, there exist three *PSA* promoter haplotypes, *−252G/−232ΔA/ARE1A*, *−252G/−232ΔA/ARE1G*, and *−252A/−232A/ARE1G*, which we have designated as *PSA*1*, *PSA*2*, and *PSA*3*, respectively (Fig. 1), according to a recently recommended nomenclature system (7). Ethnic-specific haplotype frequencies are given in Table 3.

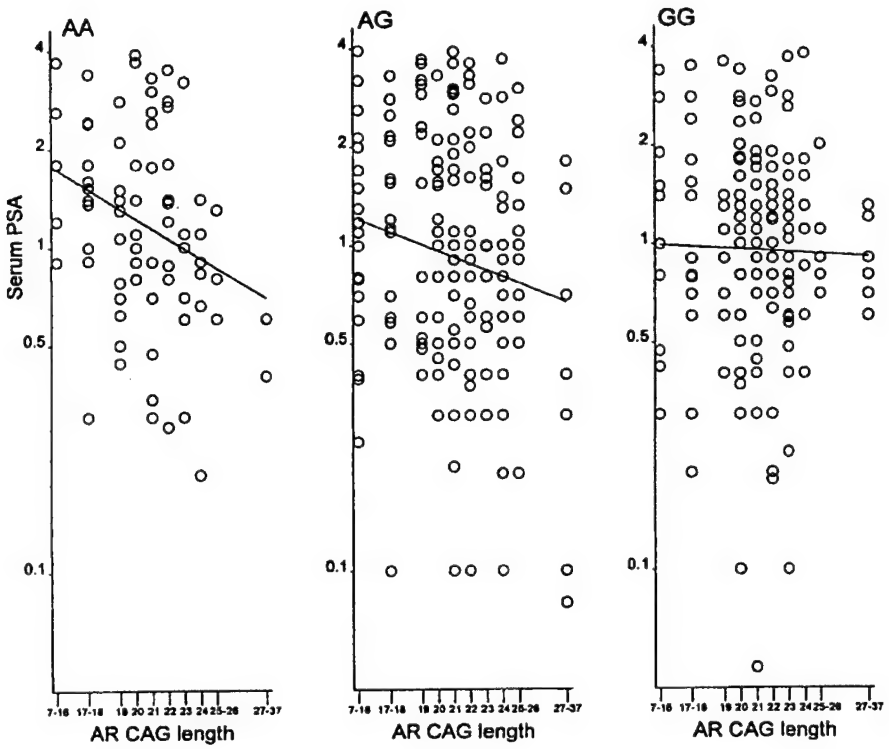


Fig. 4. Serum PSA as a function of *AR CAG* length, stratified by *PSA* genotype. O, a single subject. *Line*, regression of ln(PSA) on *AR CAG* length, adjusted for age and ethnicity. *A*, *PSA* genotype *AA*; *B*, *PSA* genotype *AG*; *C*, *PSA* genotype *GG*.

Of the two *AR* polymorphisms, only *CAG* length was associated with serum PSA levels. Serum PSA is shown as a function of *AR CAG* length in Fig. 2. There was a subtle but significant ($P = 0.01$) decrease in serum PSA with increasing *CAG* length. Serum PSA was predicted to decrease by 3.5% for each additional *CAG*. In the lowest decile (*CAG* < 17), geometric mean PSA was 83% higher (1.19 ng/ml) than in the highest decile (*CAG* > 26; geometric mean PSA, 0.65 ng/ml). There was no evidence of heterogeneity in slope by ethnicity ($P = 0.33$). There was no association between serum PSA level and *GGC* length, either before ($P = 0.25$) or after ($P = 0.29$) adjusting for *CAG* length.

Fig. 3 shows the distribution of serum PSA by ethnicity and by *PSA ARE1* genotype. Combining all ethnic groups, geometric mean PSA levels were higher among men with the *AA* genotype compared with men with the *AG* or *GG* genotypes ($P = 0.02$). PSA levels did not differ between genotypes *AG* and *GG* ($P = 0.80$). This same pattern was seen among African Americans ($P = 0.06$), non-Hispanic whites ($P = 0.28$), and Hispanics ($P = 0.11$) but was not statistically significant within each group, because of the smaller sample. The association of the *AA* genotype with higher PSA levels could not be evaluated among Japanese Americans ($P = 0.85$) because only five Japanese Americans had the *AA* genotype. Serum PSA levels were not associated with the newly identified −252*A/G* and −232*A/A* polymorphisms either alone ($P = 0.62$) or after adjusting for *ARE1* genotype ($P = 0.33$). Haplotypes (−252/−232/*ARE1*) did not predict serum PSA better than *ARE1* genotype alone ($P = 0.67$).

Because the AR regulates *PSA* transcription by binding the *ARE1* sequence, we further examined the data for evidence of interaction between *AR* and *PSA* genotypes. Stratification on *PSA ARE1* genotype (Fig. 4) provided a significantly better fit to the data than did the unstratified model (Fig. 2; $P = 0.049$). For genotype *GG*, the slope was not significantly different from zero ($P = 0.74$). For genotype *AG*, serum PSA increased 4.5% with each decrease of one *CAG* repeat decile ($P = 0.03$), whereas for genotype *AA*, serum PSA increased 7% with each decrease of one *CAG* repeat decile ($P = 0.02$).

Discussion

This is the first report to identify genetic determinants of serum PSA levels. We found that variation in serum PSA levels among healthy men is associated with polymorphic variation in both the *AR* and the *PSA* genes. Log-transformed PSA levels were linearly and inversely associated with *AR CAG* length. Moreover, *AR CAG* length influenced serum PSA most strongly among men having *PSA* genotype *AA*, only modestly among men having genotype *AG*, and not at all among men with genotype *GG*. In other words, *PSA* and *AR* genotypes interact to influence serum PSA levels.

Physical interaction of the AR transciption complex with *AREs* in the *PSA* gene promoter activates *PSA* gene transcription. *In vitro* studies have established that ARs encoded by short *CAG* alleles are more efficient transactivators than those encoded by long *CAG* alleles (8, 9). The reduction in AR transactivation activity observed *in vitro* with increasing *CAG* length is modest (10) and is consistent with the subtle decrease in serum PSA levels observed in the present study.

The *PSA ARE1* sequence lies 170 bp upstream of the transcription start site and has two allelic variants: AGAACAnnnAGTACT and AGAACAnnnAGTGCT. Experimental studies addressing the functional differences between these two alleles have not been reported. The allelic differences observed in this study were subtle and may be difficult to detect in an *in*

vitro system. Nevertheless, our data suggest that the *A* and *G* alleles interact differently with the AR, leading to quantitative differences in *PSA* expression. Alternatively, the *ARE1* polymorphism may be in linkage disequilibrium with undefined coding polymorphisms that influence *PSA* activity or with upstream or downstream regulatory elements that affect transcription efficiency. To address the possibility that the *ARE1* polymorphism may simply mark a nearby functional promoter polymorphism, we sequenced a 560-bp region surrounding the *ARE1*. Although we found two additional polymorphic sites, these sites do not appear to influence serum PSA levels.

Although PSA has been used as a tumor marker for many years, the role of PSA in prostate physiology is still unclear. Both protective and pathogenic functions have been attributed to PSA. PSA cleaves the major IGF-binding protein, IGFBP-3, and increases bioavailable IGF-I and IGF-II, potentially having a stimulatory effect on prostatic epithelial cell proliferation (11). On the other hand, PSA has been reported to be antiangiogenic (12). This function could help prevent progression of localized prostate cancers to a more advanced stage. In our previous study (5), we found that the PSA *GG* genotype, which is associated with lower serum PSA levels in the present study, was associated with increased risk of advanced prostate cancer. Although the number of subjects in that study was small, the result supports a protective role for PSA against prostate cancer progression.

The role of the *AR CAG* repeat polymorphism is less clear. The genotype associated with higher serum PSA levels, namely *CAG* short, was associated with increased risk of prostate cancer in our previous study (5) and in several other studies (13–15). This apparent inconsistency might be explained by multiple downstream effects of androgen signaling. The AR, by transactivating other genes in addition to *PSA*, might influence prostate cancer risk through several pathways, some that confer risk and others, such as PSA, that are protective. Both the *AR CAG* and the *PSA ARE1* polymorphisms need to be examined in large numbers of advanced and localized prostate cancer cases and controls to shed light on this situation.

One strength of this study is that subjects were chosen from a well-characterized cohort of healthy men. Men with elevated PSA levels (>4 ng/ml) were excluded. The remaining men are unlikely to have significant disruption of prostatic barriers; thus, differences in serum PSA levels are likely to be attributable to PSA production. Higher PSA production among certain genotypic groups might be attributable to either increased production by individual cells or to prostatic hyperplasia. We cannot rule out the possibility that the higher PSA levels among men with short *CAG* alleles might be attributable to an increase in benign prostatic hyperplasia among this group. However, our results were not changed by eliminating 40 men who reported a history of prostate enlargement. Additional studies will be necessary to determine whether intraprostatic *PSA* expression is associated with genotype.

In summary, we have shown that in healthy men, genetic variants in the *PSA* and *AR* genes contribute to variation in serum PSA levels. Men with the *PSA AA* genotype and short *AR* alleles have, on average, higher serum PSA levels.

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Androgen Receptor Activity at the Prostate Specific Antigen Locus: Steroidal and Non-Steroidal Mechanisms

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Abstract

Ligand-activated androgen receptors (ARs) occupy target genes and recruit histone modifiers that influence transcriptional competency. In LNCaP prostate cancer cells, the natural ligand 5α-dihydrotestosterone (DHT) activates transiently transfected AR-responsive promoter constructs; concurrent treatment with the protein kinase A activator forskolin enhanced AR stimulation induced by DHT. Additional treatment with the cytokine IL-6, purportedly an AR activator, markedly inhibited receptor activity. To assess AR activity on natural chromatin-integrated promoters/enhancers, we determined AR occupancy of the endogenous prostate specific antigen (PSA) promoter/enhancer as well as PSA expression in LNCaP cells treated with DHT; AR occupancy of the PSA enhancer was rapid (within 1 h of stimulation), robust (10-fold over background), and sustained (8–16 h). In contrast, AR occupancy of the PSA promoter was only increased by 2-fold. Histone H3 acetylation at both the enhancer and promoter was evident 1–2 h after DHT treatment. Detectable pre- and mature PSA mRNA levels appeared after 1 and 6 h treatment, respectively. Substantial qualitative and quantitative differences in PSA expression and AR occupancy of the PSA enhancer were observed when DHT-induced and ligand-independent activations of the AR were compared; forskolin stimulated PSA mRNA and protein expression, whereas IL-6 inhibited both DHT- and forskolin-stimulated expression. IL-6 did not diminish DHT-dependent AR occupancy of the PSA enhancer but inhibited CBP/p300 recruitment, histone H3 acetylation, and cell proliferation. These findings provide a contextual framework for interpreting the contribution of non-steroidal activation of the AR to signaling *in vivo*, and have implications for prostate cancer cell growth.

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Introduction

Androgen receptor (AR) activity is implicated in all phases of prostate cancer (1–3) including the final stages of the disease that ensue following failure of androgen ablation therapy which frequently is termed androgen independent. Recent evidence suggests that prostate cancer cells surviving after androgen ablation therapy are not necessarily resistant to subsequent alternative hormone manipulations that depend on a functional AR. Continued signaling of the AR in a castrate hormone environment could result from overexpression of the receptor, gain-of-function AR gene somatic mutations, AR coactivator overexpression, and ligand-independent activation of the AR (1, 3). It was recently proposed that stimulation of the Janus kinase-signal transducers and activators of transcription (JAK-STAT) and/or mitogen-activated protein kinase (MAPK) pathways by interleukin-6 (IL-6) and forskolin activated the AR in the absence of ligand in LNCaP prostate cancer cells (4, 5). Mechanisms for this possibly include phosphorylation of the steroid receptor coactivator-1 (6) and/or recruitment of the coactivator p300 (7). While these mechanisms theoretically may account for the continued activity of the androgen-signaling axis following androgen ablation, their contribution to AR signaling and prostate cell growth *in vivo* is not known.

The newly synthesized AR resides in the cell cytoplasm associated with a multi-protein chaperone complex essential for receptor maturation and the acquisition of ligand binding competence (8). Following binding of the native ligand, 5α-dihydrotestosterone (DHT), the multiprotein receptor complex, dissociates and the receptor is rapidly translocated into the nucleus (9) where it dimerizes and binds in the major groove of the DNA double helix to specific DNA sequences called androgen response elements (AREs). Subsequent transactivation or repression of target genes depends on the relative abundance of specific cofactors (coactivators and corepressors) in a given cell type; coactivators recruit histone acetyl transferases and certain methyl transferases, whereas corepressors recruit histone deacetylases to the transcription complex resulting in chromatin decondensation (activation) and condensation (inhibition), respectively (reviewed in Ref. 10). In this model, AR-induced activation *versus* repression of the transcription of a particular target gene is a function of the relative amount and type of cofactors recruited to the ARE receptor complex.

The expression of prostate specific antigen (PSA) is dependent on androgen signaling in prostate epithelial cells and has been used extensively as a marker of prostate cancer growth. The binding of transcription factors to two upstream *cis* regions, a proximal promoter and a distal enhancer, of the PSA gene results in transcriptional regulation. Two AREs are located

in the promoter and six are located in the enhancer region some 4.2 kb upstream from the transcription start site (11–13). Recently, chromatin immunoprecipitation (ChIP) experiments have revealed that AR complexes with both the PSA promoter and enhancer to mediate PSA expression via a ligand-dependent mechanism (14). Because most of the work assessing androgen-independent activation of the AR thus far has relied on transiently transfected reporter genes, we developed a model system for androgen-dependent and -independent activation of the AR by targeting the endogenous chromatin-integrated PSA gene and measuring its mRNA expression by reverse transcription (RT)-PCR and promoter/enhancer occupancy by ChIP analyses in LNCaP cells. Using this model system, we examined AR-mediated PSA expression and promoter/enhancer occupancy after AR activation with the natural ligand DHT, the cytokine IL-6, and the adenylate cyclase activator forskolin, and unexpectedly found that IL-6 opposed the effects of DHT and forskolin on PSA expression.

Results

AR Transactivation Activity on Transiently Transfected AR Responsive Promoter-Luciferase Constructs

Neither IL-6 nor forskolin treatment was able to stimulate the activity of any of three different androgen responsive luciferase reporter plasmids containing either a PSA promoter/enhancer (PSA-luc), a probasin promoter fragment (Probasin-luc), or an MMTV-LTR (MMTV-luc) in transiently transfected LNCaP cells (Fig. 1A). Forskolin treatment in the presence of DHT, however, resulted in a dramatic increase in activity from all three promoters, and this activity was significantly inhibited by the inclusion of IL-6 (Fig. 1B). IL-6 inhibited the DHT-mediated activity of PSA-luc, had no effect on the DHT-mediated activity of Probasin-luc, and stimulated the DHT-mediated activity of MMTV-luc (Fig. 1B).

Establishment of an Endogenous AR Reporter System

AR-mediated endogenous PSA promoter activity can be synchronized and monitored using ChIP and RT-PCR assays. This is achieved by starving the cells of steroids in growth medium containing charcoal-stripped serum, followed by the addition of the natural ligand, DHT, or alternative AR activators. The AR can bind to *cis* elements (AREs) found in the promoter and enhancer regions upstream of the transcription start site of the PSA gene (diagrammatically represented in Fig. 2A). Treatment of LNCaP cells with 10 nM DHT resulted in rapid occupancy of the PSA enhancer by the AR with maximum levels (on average some 10-fold higher than the minus antibody or minus DHT controls) being attained during the first 4 h (Fig. 2B). Acetylated histone H3 (AcH3) immunoprecipitation increased in a quantitatively similar fashion. The AcH3 time course is delayed, however, by about 1–2 h relative to AR occupancy, which is consistent with a mechanism by which the AR recruits histone acetyl transferases to the complex to allow histone H3 modification. The situation on the PSA promoter, by contrast, is different (Fig. 2B). AR occupancy is barely raised to levels 2-fold over the no antibody or minus DHT controls, whereas AcH3 levels increased quickly to about 5-fold over the no antibody or minus DHT controls, and this level was maintained for at least 16 h. The reason for the observed difference between AR occupancy on the PSA promoter and enhancer is unclear but might be related to multiple putative AREs found in the enhancer. Three different anti-AR antibody preparations raised against different epitopes gave similar results in ChIP assays (data not shown) making it unlikely that specific AR epitope masking occurred on the promoter. PCR analysis of a negative control (3′-irrelevant DNA about 6kb downstream) yielded negligible values (Fig. 2B).

PSA Expression

PSA pre-mRNA was detectable by real-time RT-PCR immediately after AR enhancer occupancy, the level increasing steadily over the following 16 h (Fig. 3A). The mature mRNA (*i.e.*, spliced) time course lagged behind that of PSA pre-mRNA with processed PSA mRNA only being detectable after about 6 h at which time it increased linearly (Fig. 3A). IL-6 treatment of LNCaP cells inhibited DHT-stimulated (Fig. 3B) and forskolin-stimulated (Fig. 3C) PSA mRNA synthesis.

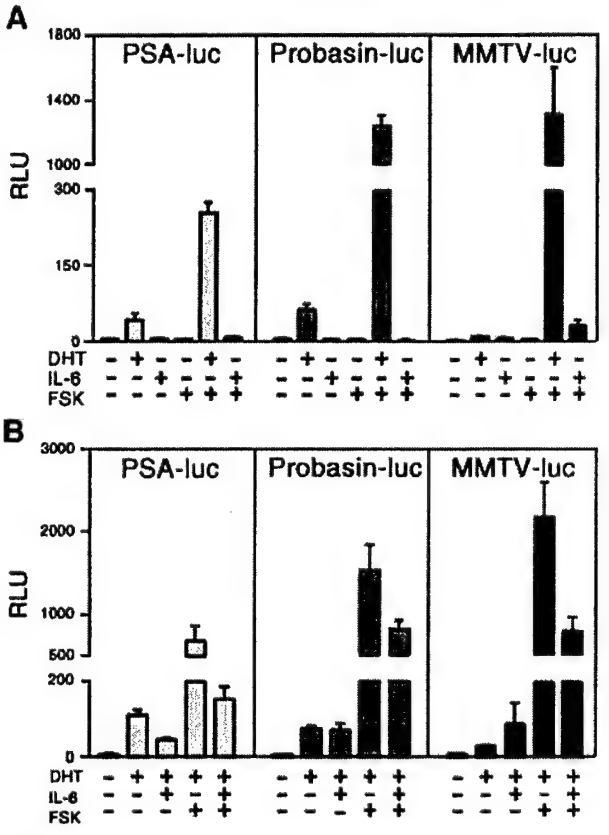

FIGURE 1. Effects of DHT, IL-6, and forskolin on transiently transfected PSA-luc, Probasin-luc, and MMTV-luc activity in LNCaP cells. LNCaP cells were transiently transfected with PSA-luc, Probasin-luc, or MMTV-luc (100 ng/well) and incubated with DHT (10 nM) and/or IL-6 (50 ng/ml) and/or forskolin (*FSK*, 50 μM) for 30 h. **A** and **B** are two independent experiments. *Columns*, means of determinations from quadruplicate wells; *bars*, SD. *RLU*, relative luciferase units.

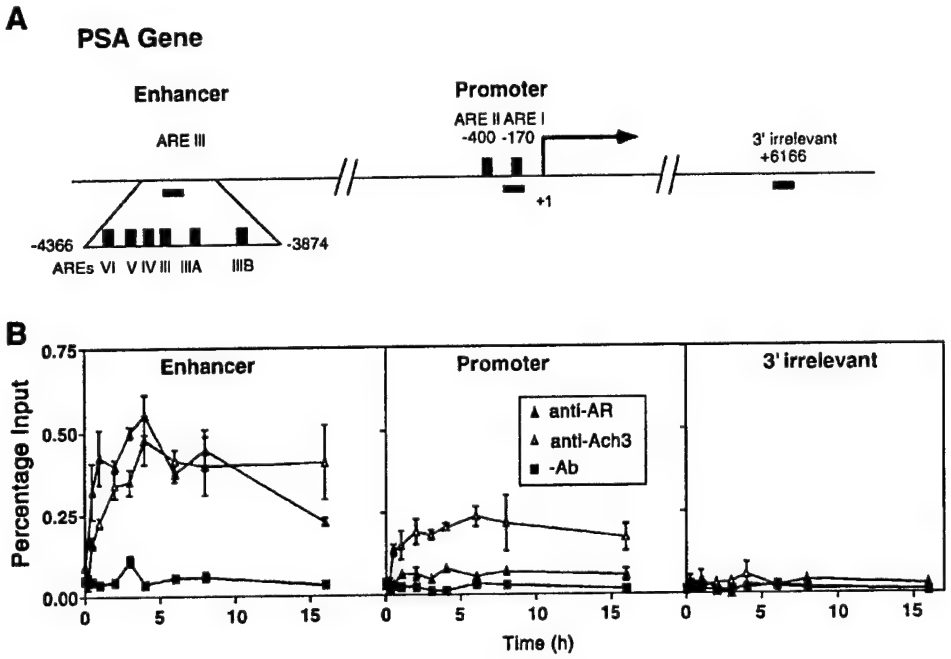

FIGURE 2. The recruitment of AR to the enhancer and promoter sequences of the PSA gene by DHT. **A.** A schematic representation is depicted of the PSA promoter and enhancer regions. *Vertical dark bars,* approximate locations of AREs. *Horizontal dark bars,* real-time PCR targeted regions. *Arrow,* transcription start site. **B.** LNCaP cells (7×10^6 cells/150 mm dish) were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 5 days and then treated with DHT (10 nM) for various times as indicated. The occupancy of AR and AcH3 on PSA gene enhancer, promoter, and 3'-irrelevant region were examined. Values are presented as percentage input. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed.

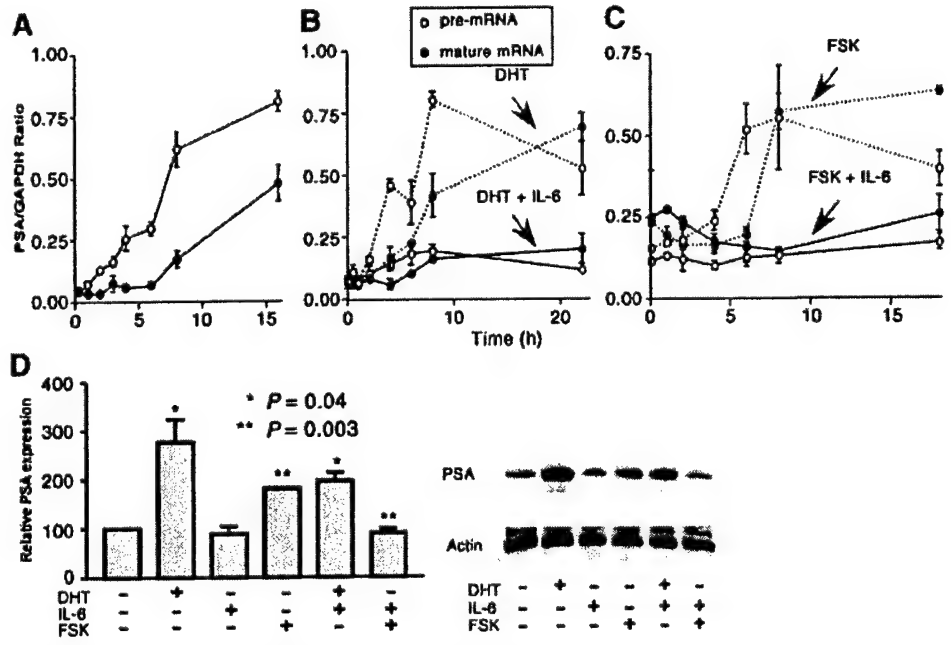

FIGURE 3. IL-6 inhibits PSA expression in LNCaP cells. LNCaP cells (2×10^6) were seeded in 100-mm dishes in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS and grown for 4 days. **A.** Cells were treated with DHT (10 nM) for various times as indicated. Real-time RT-PCR was conducted to measure the levels of pre-mRNA (*open symbols*) and mature mRNA (*closed symbols*). **B.** In a separate experiment, cells were treated with DHT (10 nM) (*dotted lines*) or DHT (10 nM) + IL-6 (10 ng/ml) (*solid lines*), and analyzed as in **A** above. **C.** In a separate experiment, cells were treated with forskolin (1 μM) (*dotted lines*) or forskolin (1 μM) + IL-6 (10 ng/ml) (*solid lines*), and analyzed as in **A** above. **D.** Western analyses of endogenous PSA protein levels were conducted in LNCaP cells exposed to DHT (10 nM) and/or IL-6 (10 ng/ml) and/or forskolin (*FSK*, 1 μM) for 12 h. PSA expression values are shown as percentage of untreated controls and normalized to actin expression on the same gels. *Columns,* means of three independent immunoblots; *bars,* SD. Two-tailed *P*-values were calculated using the Student *t* test.

Immunoblot analysis of expressed PSA protein after 12 h of similar treatments (Fig. 3D) revealed that IL-6 significantly inhibited both the DHT- and forskolin-stimulated PSA protein expression consistent with the mRNA data.

IL-6 Does Not Inhibit AR Occupancy of the PSA Enhancer

In an attempt to understand the mechanism of IL-6 inhibition of PSA expression, we analyzed AR occupancy and histone H3 acetylation of the PSA enhancer (Fig. 4). IL-6 had little effect on DHT-mediated AR occupancy but failed to stimulate histone H3 acetylation (Fig. 4A), a result consistent with the inhibitory effects of IL-6 on PSA expression. In the absence of DHT, no significant AR localization on the PSA enhancer was observed after either IL-6 or forskolin treatment (data not shown). Because CBP/p300 has intrinsic histone acetyl transferase activity and is often recruited to the transcription complex by steroid receptors, we analyzed for its presence on the PSA enhancer/promoter by ChIP. DHT-mediated recruitment of CBP/p300 was inhibited by IL-6 on both the PSA promoter and enhancer (Fig. 4B).

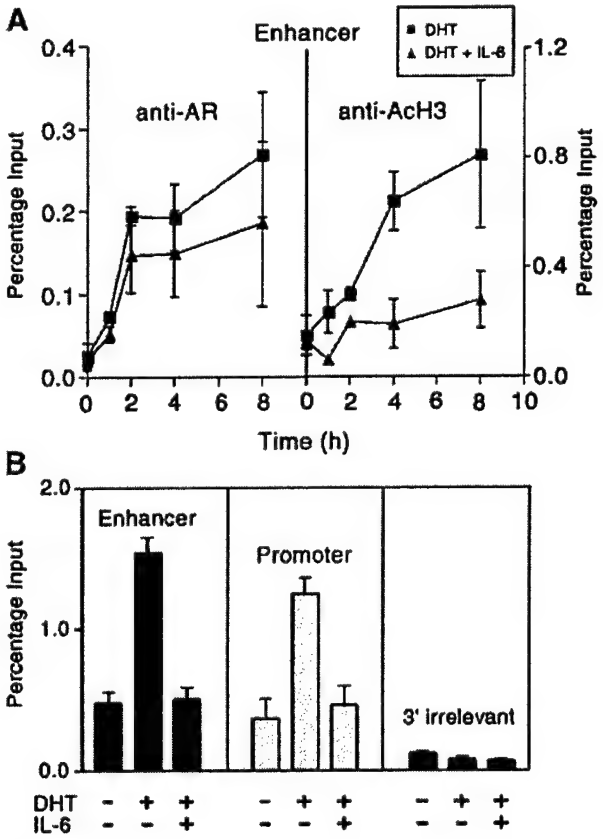

FIGURE 4. Effect of IL-6 on AR occupancy and recruitment of CBP/p300. **A.** LNCaP cells (7×10^6 cells/150 mm dish) were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days and then treated with DHT (10 nM) (■) or DHT (10 nM) + IL-6 (10 ng/ml) (▲) for various times as indicated. The occupancy of AR and AcH3 on the PSA gene enhancer were examined. **B.** LNCaP cells (7×10^6 cells/150 mm dish) were treated as in **A** for 1 h. CBP/p300 occupancy on the PSA gene enhancer, promoter, and 3′-irrelevant regions at the PSA locus was examined.

IL-6 Inhibits DHT- and Forskolin-Stimulated Cell Proliferation

Consistent with the AR activity as measured on the PSA promoter/enhancer (see above), IL-6 significantly inhibited both the DHT- and forskolin-stimulated increases in cell number after 3 days of treatment (Fig. 5).

Discussion

Novel findings of this study include the significantly higher DHT-induced AR occupancy of the PSA enhancer compared to the PSA promoter, and the opposing activities of the non-steroidal activators, forskolin and IL-6, at the same locus. These findings were made possible by the development of an *in vivo* system to determine ligand-dependent and -independent activity of the AR. It employs endogenous, chromatin-integrated PSA gene expression as a reporter in LNCaP cells, coupled with quantitative assessments of DNA occupancy by proteins using ChIP assays. Since most of the previous studies on AR activation have relied on functional and interaction assays performed in transient transfection systems, a more physiological system was sought. The ChIP assay provides a method to interrogate the molecular composition of complexes on natural promoters *in vivo*. In the case of steroid receptors, promoter occupancy can be synchronized by the addition of ligand to the cultures. As a result, occupancy can be assessed as a function of both time (*i.e.*, kinetically) and composition by using appropriate antibodies. Multiple protein-protein interactions are likely to be required to recruit AR and its associated proteins in the appropriate conformation to initiate transcription via the basal transcription machinery. In addition, ligand-independent activation of the AR may differ from ligand-dependent activation in both promoter and cell contexts. For example, ligand-independent activation may cause the AR to adopt a different conformation than that assumed by the ligand-bound AR, which in turn could result in exposure of different protein-protein interaction surfaces in the AR leading to the recruitment of different coregulator proteins and altered activation of AR regulated genes.

In the present study, we observed quantitatively and qualitatively different results with the natural ligand, DHT, and the putative ligand-independent activators of the AR, forskolin and IL-6, depending on the promoter context and whether we measured endogenous or transiently transfected gene expression. Forskolin treatment increased endogenous PSA expression, but not the expression from transiently transfected reporters in the absence of DHT. IL-6, on the other hand, increased transiently expressed reporters in the presence of DHT from some but not all promoters and inhibited both DHT- and forskolin-mediated expression from endogenous and some transiently expressed reporters. These results might be explained by the differential recruitment of coactivators and corepressors (10) to *cis*-regulatory regions in the transiently transfected plasmids and chromatin-integrated genes. AR-mediated coactivator recruitment has been extensively studied and includes p160 coactivator interactions with both the AR ligand-binding domain and the carboxy-terminal portion of the AR amino-terminal domain (15, 16). The p160 coactivators recruit secondary coactivators via two activation domains,

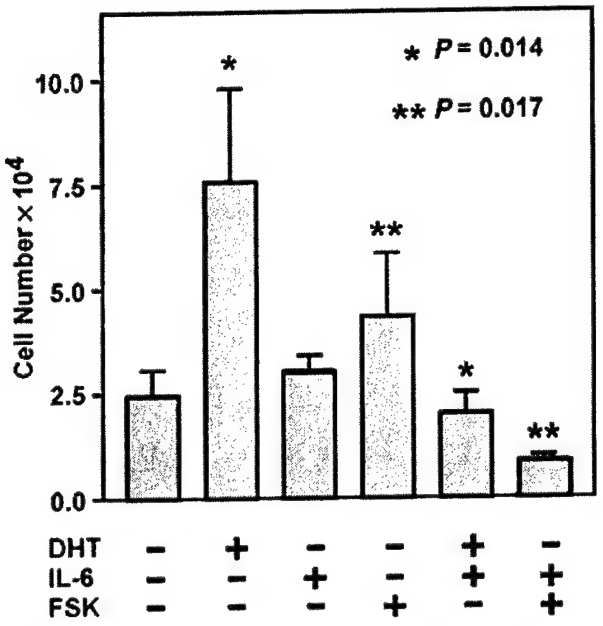

FIGURE 5. Effect of IL-6 on LNCaP cell proliferation. LNCaP cells (2×10^4 cells/16 mm cluster well) were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days and then treated with DHT (10 nM) and/or IL-6 (10 ng/ml) and/or forskolin (*FSK*, 1 μM) for 3 days as indicated. Cells were then trypsinized and counted in a hemocytometer. *Columns*, means of triplicate wells; *bars*, SD. Two-tailed *P*-values were calculated using the Student *t* test.

AD1 and AD2. AD1 binds CBP/p300, which possesses intrinsic histone acetyl transferase activity, whereas AD2 recruits the coactivator-associated arginine methyltransferase (17). AR-mediated corepressor recruitment has recently been observed with SMRT interacting with the AR amino-terminal domain (18) and NCoR with the AR ligand-binding domain (19).

Using quantitative real-time PCR assessments of the amount of DNA recovered after immunoprecipitation of cross-linked chromatin, we examined the kinetics of AR occupancy on the PSA enhancer and promoter. AR occupancy of the PSA enhancer was rapid (within 1 h of stimulation), robust (10-fold over background), and sustained (8–16 h). In contrast, AR occupancy of the PSA promoter was only 2-fold over background. No evidence of significant cycling of the AR on and off the PSA promoter and enhancer, as previously reported for the ER (20) and AR (14, 21), was observed. This may reflect the quantitative nature of the present ChIP analysis. Previous studies used non-quantitative end-point assessments of PCR bands on agarose gels, which may have exaggerated small differences in amounts of target DNA and thus not reflected the true situation *in vivo*.

Forskolin, which has previously been shown to activate the AR through a PKA-stimulated pathway involving the amino terminus of the receptor (reviewed in Ref. 22), activated PSA promoter activity independently from DHT in our endogenous system, and only in the presence of DHT in the transiently transfected system. In LNCaP cells, forskolin most likely increases MAPK activity via PKA stimulation (23). Inhibitors of MAPK or a mutation of the AR (S513A), which is located in a consensus MAPK phosphorylation site in the AR-NTD, abrogate AR transactivation activity in transient transfection assays in the presence of low concentrations of androgen (24, 25). Taken together, MAPK-mediated activity most likely contributes to ligand-independent AR activation and might be a major signaling mechanism in hormone refractory prostate cancer.

IL-6, a M_r 21,000–28,000 cytokine, has been proposed as a contributor to prostate cancer progression (reviewed by Smith *et al.* 26). Interestingly, IL-6 had opposite growth effects on prostate cancer cells depending on whether they are androgen dependent or refractory. Chung *et al.* (27) compared a series of hormone-dependent and -refractory cell lines with respect to IL-6 secretion and growth and concluded that IL-6 might undergo a transition from a paracrine growth inhibitor to an autocrine growth stimulator during the progression of prostate cancer to become hormone refractory. Similarly, Hobisch *et al.* (28) found that long-term treatment of LNCaP cells with IL-6 led to abolishment of its inhibitory growth response. In an earlier study, IL-6 inhibited the stimulatory action of androgens on apolipoprotein D and gross cystic disease fluid protein 15 (both known AR target genes) expression in human breast cancer cells (29). Taken together with the results in the present study, where we have demonstrated unequivocally that IL-6 inhibited DHT- and forskolin-stimulated gene expression and cell proliferation, they suggest that long-term exposure of hormone-dependent prostate epithelial cells to IL-6 will shift its effect on the AR from inhibitory to stimulatory depending on the balance of coactivators/corepressors recruited to the AR. If this indeed is the case, inflammatory responses in the prostate mediated by IL-6 secretion might have initial benefits, but later deleterious effects with respect to prostate cancer growth and progression.

Whereas IL-6 and forskolin potentially can activate the MAPK signaling pathway, it appears that their effects on PSA expression in LNCaP cells occur via different mechanisms. In our system, IL-6 and forskolin had opposite effects on endogenous PSA expression. It is possible that the inhibitory effects of IL-6 observed in our study are mediated via non-MAPK mechanisms such as the JAK-STAT pathway. The MAPK and JAK-STAT pathways might therefore affect AR and/or coactivator and/or corepressor phosphorylation in different ways to elicit the opposite effects on AR-mediated gene expression. The challenge remains to disentangle such mechanisms to understand maintenance of AR activity in the prostate in a castrate or androgen ablated state. The endogenous PSA promoter/enhancer system described here provides a model system for such an elucidation.

Materials and Methods

Cell Culture and Materials

Human prostate cancer LNCaP cells obtained from the American Type Culture Collection (Manassas, VA) (ATCC CRL-1740) were maintained in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 5% (v/v) heat-inactivated fetal bovine serum (FBS) and were used between passages 25–70 at a split ratio of 1:4 at each passage. DHT and forskolin were purchased from Sigma Chemical Co. (St. Louis,

MO). Recombinant human IL-6 was obtained from R&D Systems, Inc. (Minneapolis, MN). Antibodies were anti-AR (N20) and anti-actin (Santa Cruz Biotechnology, Santa Cruz, CA), anti-PSA (DAKO Corp., Carpinteria, CA) and anti-AcH3, and anti-CBP/p300 (Upstate Biotechnology, Inc., Lake Placid, NY).

Transient Transfection and Luciferase Detection

LNCaP cells (5×10^5 cells/well) were plated in 96-well plates and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS (Gemini, Woodland, CA) for 3 days. Cells were then transfected with reporter PSA-luc (pGL3_PSA540), Probasin-luc (ARR3-tk-luc/tk81-PB3), or mouse mammary tumor virus-luciferase (MMTV-luc) plasmid DNA (100 ng/well) using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. pGL3-PSA540-enhancer (PSA-luc) is a mammalian expression vector that contains firefly luciferase linked to the androgen responsive 548-bp PSA promoter region (−541 to +7) preceded by 1450 bp of the PSA enhancer region (−5322 to −3873) provided by Bristol-Myers Squibb (Princeton, NJ). ARR3-tk-luc/tk81-PB3 (Probasin-luc) is a mammalian expression vector that contains firefly luciferase linked to three copies of the androgen responsive minimal rat probasin promoter (−244 to −96) ligated in tandem to the thymidine kinase (tk) enhancer element (30, 31) provided by Dr. R.J. Matusik (The Vanderbilt Prostate Cancer Center, Nashville TN). MMTV-luc is a mammalian expression vector that contains firefly luciferase linked to MMTV long terminal repeats (LTR) with four hormone responsive elements provided by Dr. R.M. Evans (The Salk Institute, La Jolla, CA). After transfection, cells were grown in phenol red-free RPMI 1640 containing 0.5% charcoal/dextran-stripped FBS with DHT and/or IL-6 and/or forskolin as indicated. After additional 30 h incubation, the cells were lysed with the passive lysis buffer (Promega, Madison, WI). The extracts were assayed for luciferase activity using the Promega assay kit according to the manufacturer's protocol and measured on a Dynex MLX Microtiter Plate Luminometer (Chantilly, VA). Relative luciferase units (RLU) are shown as the means ± SD of quadruplicate wells. Total protein concentrations of the extracts were assayed using Bio-Rad Protein Assay Kit (Hercules, CA) according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices EMax Microplate Reader (Sunnyvale, CA). No significant differences in total protein concentrations were observed among the different wells within a given experiment.

ChIP Assays

LNCaP cells (7×10^6 cells/150 mm dish) were plated and grown in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS for 5 days. Cells were treated with DHT and/or IL-6 and/or forskolin for various times as indicated, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The dishes were rinsed twice with ice-cold PBS, and the cells were scraped into PBS containing protease inhibitors (Sigma) and centrifuged for 3 min at 2000 rpm. The cell pellets were resuspended in 0.35 ml lysis buffer [1% SDS, 10 nM EDTA, 50 nM Tris-HCl (pH 8.0), 2× protease inhibitors] and incubated for 10 min on ice. The resulting cell lysates were sonicated in 1.5 ml microfuge tubes for 5 s, followed by a 1-min incubation on ice for a total of four cycles using setting 10 of a Fisher Sonic Dismembrator Model 60 (Tustin, CA). Cell debris was removed by centrifugation for 10 min at 14,000 rpm in a microfuge. The soluble chromatin (100 µl per immunoprecipitation of the supernatant) was transferred to a new microfuge tube and diluted 10-fold in dilution buffer [0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl (pH 8.0), 167 mM NaCl, protease inhibitors]. The diluted suspension (1 ml) was precleared by incubating with 2 µl sheared salmon sperm DNA (1 µg/µl) (Life Technologies) and 45 µl protein G-Sepharose bead suspension (Amersham Pharmacia Biotech, Piscataway, NJ) [50% slurry in 10 mM Tris-HCl (pH 8.0), 1 mM EDTA] for 1 h at 4°C with rotation. The supernatant was immunoprecipitated by incubating overnight at 4°C with the indicated specific antibodies. The next morning, 2 µl sheared salmon sperm DNA and 45 µl protein G-Sepharose were added for 1 h at 4°C with rotation. The Sepharose-bound immune complexes were sequentially washed for 10 min each in a low salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl], a high salt wash buffer [0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl], and a LiCl wash buffer [0.25 M LiCl, 1% IGEPAL CA-630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)] with rotation. The Sepharose-bound immune complexes were further washed twice with TE buffer [10 nM Tris-HCl, 1 mM EDTA (pH 8.0)]. The immune complexes were recovered from the beads with an elution buffer (1% SDS and 0.1 M $NaHCO_3$). Eluates were heated at 65°C for 4 h to reverse the formaldehyde cross-links. DNA was purified by phenol/chloroform extraction and ethanol precipitation. The DNA pellets were finally resuspended in 100 µl H_2O ready for real-time PCR analysis (see below). Ten percent input samples were treated in the same way except that no immunoprecipitation steps were performed.

Real-Time PCR of DNA Obtained From ChIP Assays

DNA samples from ChIP preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad) and AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ). The primers and probes were: enhancer forward, 5′-GCCTGGATCTGAGAGAGATATCATC-3′; reverse, 5′-ACACCTTTTTTTTTCTGGATTGTTG-3′; promoter forward, 5′-CCTAGATGAAGTCTCCATGAGCTACA-3′; reverse, 5′-GGGAGGGAGAGCTAGCACTTG-3′; 3′-irrelevant locus forward, 5′-TCATCATGAATCGCACTGTTAGC-3′; reverse, 5′-GCCCAAGTGCCTTGGTATACC-3′; enhancer probe, 5′-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3′; promoter probe, 5′-6-FAM-CAATTACTAGATCACCCTG-GATGCACCAGG-BHQ-1-3′; and 3′-irrelevant locus probe, 5′-6-FAM-TGAATCATCTGGCACGGCCCAA-BHQ-1-3′ (Biosearch Technologies, Novato, CA). Triplicate PCR reactions for each sample (5 µl) were conducted. The results are given as percentage of input and represent mean values ± SD of triplicate determinations.

Real-Time RT-PCR of RNA

After treatment of the cells with DHT or forskolin and/or IL-6, total cellular RNA was prepared and treated with RNase-free DNase I using the SV Total RNA Isolation System (Promega). A two-step RT-PCR method was employed using the TaqMan Gold RT-PCR Kit (Applied Biosystems). In these analyses, the same probe and reverse primer located in exon 4 of PSA gene were used for both the pre- and mature mRNA determinations (see below). The forward primer is located in intron 3 for pre-mRNA and in exon 3 for mature mRNA determinations. The size of RT-PCR product was checked by agarose gel electrophoresis (data not shown), whereas mRNA quantitation was performed by real-time RT-PCR. Thus, the primers and probes were: PSA pre-mRNA forward, 5′-GTTTTTGCCTGGCCC-GTAG-3′; mature mRNA forward, 5′-GGCAGCATTGAACCAGAGGAG-3′; PSA reverse, 5′-GCATGAACTTGGTCACCTTCTG-3′; PSA probe, 5′-6-FAM-ATGACGTGTGTGCGCAAGTTCACCC-BHQ-1-3′; GAPDH forward, 5′-GTC-A-TGGGTGTGAACCATGAGA-3′; GAPDH reverse, 5′-GGTC-ATGAGTCCTTCCACGATAC-3′; and GAPDH probe, 5′-6-FAM-CAGCCTCAAGATCATCAG-CAATGCCTC-BHQ-1-3′ (Biosearch Technologies). Triplicate PCR reactions were conducted. GAPDH mRNA expression was analyzed in each sample in parallel wells. The results are represented as PSA/GAPDH mRNA ratios. Due to extensive DNase treatment of the RNA preparations (total RNA Isolation System; Promega), no significant genomic DNA contamination was apparent; (*a*) negative controls lacking reverse transcriptase were normally less than 1% of the experimental values (the value of each sample was adjusted by subtracting these negative values), and (*b*) PCR analyses of promoter sequences gave negligible values.

Immunoblotting

LNCaP cells (6×10^5 cells/60 mm dish) were plated and grown in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped FBS for 3 days. After treatment with DHT and/or IL-6 and/or forskolin as indicated, cells were harvested in 100 μl RIPA buffer [10 mM sodium phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate, 0.2 mM Na_3VO_4 (pH 7.2)] that contained a cocktail of mammalian protease inhibitors. Equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech) and probed with rabbit polyclonal anti-PSA or anti-actin antibody. HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed using the enhanced chemiluminescence Western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Autoradiograms from three independent Western gels were analyzed by scanning densitometry using a Model GS-710 Imaging Densitometer (Bio-Rad).

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Dynamic methylation of histone H3 at lysine 4 in transcriptional regulation by the androgen receptor

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ABSTRACT

The methylation of histone H3 correlates with either gene expression or silencing depending on the residues modified. Methylated lysine 4 (H3-K4) is associated with transcription at active gene loci. Furthermore, it was reported that trimethylated but not dimethylated H3-K4 is exclusively associated with active chromatin in *Saccharomyces cerevisiae*. In the present study, we investigated the H3-K4 methylation at the human prostate specific antigen (PSA) locus following gene activation and repression via androgen receptor (AR). We show that ligand-induced, AR-mediated transcription was accompanied by rapid decreases in di- and trimethylated H3-K4 at the PSA enhancer and promoter. Moreover, the observed decreases in H3-K4 methylation were reversed when AR was inhibited by a specific AR antagonist, bicalutamide. In contrast to the decreases in methylation at the 5′ transcriptional control regions of the PSA gene, H3-K4 methylation in the coding region steadily increased after a lag period of ~4 h. The results suggest a novel role of methylated H3-K4 in transcriptional regulation.

INTRODUCTION

Chromatin, a highly structured and regulated polymer of genomic DNA, histones and non-histone proteins, is the physiological template of all eukaryotic genetic information. Recent advances have firmly established that, beyond its architectural role, chromatin plays a more specific role in the regulation of gene expression. Central to this regulation is the dynamic organization and modification of nucleosomes, which are the basic repeating unit of chromatin and are comprised of 146 bp of DNA wrapped around histone octamers. Structural analysis of the nucleosome has revealed that the N-terminal tails of histones are positioned peripheral to the nucleosomal core, suggesting that they are potentially involved in protein–protein interactions (1). Indeed, histone N-terminal tails are subject to various covalent modifications such as acetylation, phosphorylation, ubiquitination and methylation by specific chromatin-modifying enzymes (2). Such modifications can determine the functional state of chromatin, and therefore affect various downstream processes, possibly by directly altering DNA–histone affinity or by providing recognition motifs for chromatin-associated proteins (3,4).

The role of methylated histone lysines in gene regulation has recently attracted considerable interest. Unlike acetylation or phosphorylation, methylation does not alter the overall charge of the N-terminal tails of histones, implying a mechanism other than regulating nucleosomal compaction via charge neutralization (5). Instead, histone methylation was reported to serve as a recognition motif for chromatin-associated proteins such as HP1, which binds to H3 methylated at lysine 9 to establish heterochromatic regions (6,7). Methylated lysines occur in mono-, di- or trimethylated states that are catalyzed by specific enzymes with varying transcriptional outcomes. For example, human SET7/9 catalyzes monomethylation of histone H3 at lysine 4 (H3-K4), whereas yeast Set1 catalyzes di- and trimethylation of H3-K4, and trimethylated but not dimethylated H3-K4 has been reported to be exclusively associated with active transcription in *Saccharomyces cerevisiae* (8,9). Furthermore, whereas acetylation and phosphorylation have been demonstrated to be reversible, methylation has been suggested to be stable and irreversible, partly due to the fact that no histone demethylase has been discovered to date (10). It is suggested, therefore, that methylation is a permanent modification that serves as a 'molecular memory of transcription' that could be heritably maintained. However, in examples where dynamic transcriptional regulation is essential, reversibility of methylation may be necessary. For example, thyroid hormone receptor (TR), which has the ability to alternately and rapidly repress or activate transcription depending on the absence or presence of cognate ligand, appears to regulate histone methylation of lysine as well as arginine residues concomitant with transcriptional activation of target genes (11).

Nuclear hormone receptors (NR) are members of a large group of structurally related transcription factors that are regulated by lipophilic ligands. The androgen receptor (AR), a member of the nuclear receptor superfamily, activates transcription of specific target genes by binding to androgen responsive elements (AREs) upstream of the transcription start site and by recruiting both coactivators and other components of the general transcriptional machinery (12).

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While transcriptional activation is driven by the binding of 5-α-dihydrotestosterone (DHT) to the AR, antagonists such as bicalutamide repress transcription through the recruitment of corepressors, SMRT and N-CoR, as well as histone deacetylases (HDACs) (13). Although histone arginine methyltransferases such as CARM1 and PRMT1 were reported to facilitate transcriptional activity of NR (14), the role of histone lysine methylation is still unclear.

In this study, we report that changes in methylated H3-K4 status occur at various loci within the human prostate specific antigen (PSA) gene during early stages of transcriptional regulation by the AR. Decreases in both di- and trimethylated H3-K4 accompanied AR binding at the enhancer and promoter and were completely reversed by the addition of an AR antagonist, bicalutamide. Conversely, substantial increases in di- and trimethylated H3-K4 were observed in the coding region of the PSA gene as a function of gene expression. Together these results suggest distinct functions conferred by histone methylation at the transcriptional control regions versus coding regions of active genes.

MATERIALS AND METHODS

Cell culture and reagents

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (Manassas, VA) and grown in RPMI 1640 (Invitrogen, Grand Island, NY) supplemented with 10% (v/v) heat-inactivated FBS (Gemini Bioproducts, Woodland, CA). DHT was purchased from Sigma-Aldrich (St Louis, MO). Bicalutamide was obtained from ICI Pharmaceuticals (UK).

Chromatin immunoprecipitation (ChIP) assays

LNCaP cells (5×10^6 cells/150 mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% charcoal/dextran-stripped FBS (Gemini Bioproducts, Woodland, CA) for 3 days. Cells were treated with DHT and/or bicalutamide for various times as indicated, cross-linked by adding formaldehyde (1%) directly to the culture medium, and incubated at room temperature for 10 min. The cells were washed twice with ice-cold PBS and harvested by scraping and centrifugation at 3000 *g* for 5 min. The cell pellets were resuspended in 0.5 ml lysis buffer [1% SDS, 10 nM EDTA, 50 nM Tris–HCl pH 8.0, with 1× complete protease inhibitor cocktail (Roche, Indianapolis, IN)] and incubated for 20 min on ice. The cell lysates were sonicated at setting 4 on a Branson Sonifier Cell Disruptor 185 for 10 s. The sonication was repeated five times (with 1 min incubations on ice in between sonications) and insoluble materials were removed by centrifugation at 15 500 *g* for 10 min. For each immunoprecipitation, 100 µl of supernatant containing soluble chromatin was diluted 10-fold in dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl pH 8.0, 167 mM NaCl, 1× protease inhibitor cocktail). After preclearing with 75 µl of protein G–Sepharose (Amersham Pharmacia Biotech, Piscataway, NJ) at 4°C for 1 h, the supernatant was immunoprecipitated by incubating at 4°C overnight with 25 µl anti-MLL, 25 µl anti-AR (N20, Santa Cruz Biotechnology, Santa Cruz, CA), 5 µl anti-dimethyl H3-K4, 5 µl anti-AcH3 (Upstate Biotechnology, Lake Placid, NY) or 5 µl anti-trimethyl H3-K4 (Abcam, Cambridge, UK). Immune complexes were obtained by incubating with 50 µl of protein G–Sepharose at 4°C for 1 h. Immunoprecipitates were sequentially washed for 5 min each in low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 500 mM NaCl), LiCl wash buffer (0.25 M LiCl, 1% IGEPAL CA 630, 1% deoxycholate, 1 mM EDTA, 10 mM Tris–HCl pH 8.0) and TE buffer (twice). Washed beads were extracted with 250 µl of elution buffer (1% SDS, 100 mM $NaHCO_3$) twice, the elution was combined, and the protein–DNA cross-linking was reversed by incubation at 65°C overnight. Each sample was treated with 20 µg of proteinase K (Gibco BRL, Grand Island, NY) in proteinase K buffer (50 mM Tris–HCl pH 6.5, 10 mM EDTA) at 45°C for 1 h. DNA was purified by phenol/chloroform extraction, precipitated by ethanol with glycogen, and resuspended in 100 µl of H_2O. One percent of total soluble chromatin was processed in parallel without immunoprecipitation, and values obtained from this DNA were used as denominators to calculate immunoprecipitated DNA as percentage of input.

Real-time PCR of ChIP DNA

Immunoprecipitated DNA was analyzed using quantitative real-time PCR as described previously (15). Briefly, triplicate PCRs for each sample were mixed with AmpliTaq Gold PCR master mix (Applied Biosystems, Branchburg, NJ), forward and reverse primers and probe to be analyzed by Bio-Rad iCycler optical system. The primers and probes were: enhancer forward, 5′-GCCTGGATCTGAGAGAGATATC-ATC-3′; reverse, 5′-ACACCTTTTTTTTTCTGGATTGTTG-3′; promoter forward, 5′-CCTAGATGAAGTCTCCATGAG-CTACA-3′; reverse, 5′-GGGAGGGAGAGCTAGCACTTG-3′; 3′ forward, 5′-TCATCATGAATCGCACTGTTAGC-3′; reverse, 5′-GCCCAAGTGCCTTGGTATACC-3′; E-P forward, 5′-CAGTGGCCATGAGTTTTGTTTG-3′; reverse, 5′-AACCAATCCAACTGCATTATACACA-3′; exon 3 forward, 5′-CACACCCGCTCTACGATATGAG-3′; reverse, 5′-GAGCTCGGCAGGCTCTGA-3′; enhancer probe, 5′-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3′; promoter probe, 5′-6-FAM-CAATTACTAGATCACC-CTGGATGCACCAGG-BHQ-1-3′; 3′ probe, 5′-6-FAM-TGAATCATCTGGCACGGCCCAA-BHQ-1-3′; E-P probe, 5′-6-FAM-CCCAACGCAACTTAACCTAACAAG-BHQ-1-3′; and exon 3 probe, 5′-6-FAM-CTCCAGCCACGACCT-CATGCTGCT-BHQ-1-3′ (Biosearch Technologies, Novato, CA). This assay provides a precise quantitation of target DNA and is based on the principle of fluorophore release from a self-quenching probe; the instrument measures the number of cycles (C_t) required for fluorescence to exceed a set threshold. A standard curve of known target DNA is constructed in parallel from which the relative amount of target DNA in the sample is calculated. Values are presented as percentage input, which is analyzed at the same time. The precise quantitative nature of this analysis is superior to analyses commonly used by others that rely on semi-quantitative end-point assessments of PCR bands on agarose gels.

Histone isolation and immunoblotting

Total histones were isolated as described (16) with the following modifications. LNCaP cells were washed once with ice-cold PBS, harvested in 500 µl PBS containing 10 mM sodium butyrate and centrifuged at 700 *g* for 1 min. The pellet was resuspended in 1 ml lysis buffer (1% Triton X-100, 8.6% sucrose, 10 mM Tris–HCl pH 6.5, 10 mM sodium butyrate, 50 mM sodium disulfite, 10 mM $MgCl_2$) and centrifuged at 1000 *g* for 1 min. The lysis was repeated three times until a white nuclear pellet was attained. After a final wash with 10 mM Tris–HCl pH 7.4, 13 mM EDTA, the pellet was resuspended in ice-cold distilled water. H_2SO_4 was added to a final concentration of 0.4 N and incubated on ice for 1 h. After centrifugation at 10 000 *g* for 5 min, the supernatant containing total histones was precipitated with 10× vol of acetone at –20°C overnight. The total histone pellet was recovered by centrifugation at 1000 *g* for 5 min and resuspended in distilled water. Total protein concentration of the isolated histones was assayed using a Bio-Rad Protein Assay Kit (Hercules, CA) according to the manufacturer's protocol, and 600 nm absorbance was measured on a Molecular Devices Emax Microplate Reader (Sunnyvale, CA). Equal amounts of total histones were analyzed by SDS-PAGE, transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and probed with the indicated antibodies. Anti-unmodified H3 (Upstate Biotechnology) were used as controls. HRP-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed using the enhanced chemiluminescence western blotting system (Amersham Pharmacia Biotech) according to the manufacturer's protocol. Images are representative of three independent immunoblots and were analyzed by Fluor-S Max MultiImager Quantification System (Bio-Rad).

Real-time RT–PCR of RNA

PSA mRNA was analyzed as described previously using quantitative RT–PCR (15).

RESULTS AND DISCUSSION

Di- and trimethylation of H3-K4 decrease during transcriptional activation by AR

In *S.cerevisiae*, the precise methylation status of histone H3-K4 was reported to determine transcriptional activity. The trimethylated but not dimethylated form of H3-K4 is preferentially associated with active transcription, and thus it was hypothesized that the dimethylated state may function as a transcriptionally permissive signal whereas the trimethylated state exclusively denotes active transcription (9). We have sought to address whether the transcriptional status conferred by H3-K4 methylation is conserved in human by investigating a nuclear hormone receptor model where transcription can be rapidly activated or repressed. To this end, we examined H3-K4 methylation in AR-mediated PSA gene transcription by utilizing a human prostate cancer cell line, LNCaP, which expresses both PSA and AR. PSA gene transcription is tightly regulated by AR. Upon ligand induction, ARs bind to AREs located in the proximal promoter and upstream enhancer of the PSA gene, and transcription is initiated following the subsequent recruitment of coactivators and RNA polymerase II (RNAP II) to both control regions (17).

In order to study AR-mediated effects at target gene loci, we quantitated specific histone modifications as well as AR occupancy using ChIP analysis and specific real-time PCR primers that span various loci in the PSA gene (Fig. 1A). Specifically, we examined the proximal promoter, the distal enhancer (~4.1 kb upstream of the transcription start site), and exon 3 (~3.2 kb downstream of the transcription start site). As controls, we also designed primers at a region between the enhancer and promoter (E-P) and a region 3′ to the poly-A addition site (3′). AR occupancy followed a previously described (15) pattern at the enhancer and promoter with the quantitative difference possibly due to the presence of multiple AREs at the enhancer (Fig. 1C). AR occupancy was essentially absent in the three loci without AREs. Acetylation of H3 (K9 and K14) increased at the enhancer, promoter and exon 3 but not at either E-P or 3′ (Fig. 1D). As with AR occupancy, acetylation also displayed a quantitative difference between the enhancer and promoter, with a significantly higher level at the enhancer. This may be due to the multiple bound-ARs recruiting a proportional number of histone acetyltransferases (HATs) at the enhancer. In support, a recent study demonstrated that p160 coactivators and CBP/p300 HATs are preferentially recruited to the enhancer (17). Also, biochemical and genetic studies have revealed that the enhancer is required for maximal PSA expression and thus, together, establish the enhancer as a crucial control region (18). Interestingly, acetylation at the coding region was higher than either at the enhancer or promoter, indicating that acetylation is not limited to the transcriptional control regions and may play a significant role in elongation. *In vitro*, nucleosomes present a considerable obstacle to the elongating RNAP II causing transcription inhibition, which can be relieved by acetylation (19,20). Furthermore, RNAP II association is reduced by hypoacetylation in the coding regions in yeast (21). Thus, acetylation may be important in transcription elongation as well as initiation.

Strikingly, both di- and trimethylated H3-K4 decreased with ligand induction at the enhancer and promoter, but not at the other three loci (Fig. 1E and F). The observation that both di- and trimethylated H3-K4 are decreased cannot be explained by the possibility that further methylation of dimethylated lysine occurred to form trimethylated lysine, or that trimethylated lysine was demethylated to form dimethylated lysine. To address the concern that trimethyl H3-K4 antibody (Abcam) may cross-react with methylated H3-K9, we analyzed for the presence of methylated H3-K9 with the specific antibody (Upstate) in ChIP assays and found that methylated H3-K9 was essentially absent at the PSA gene (data not shown). Thus, the decrease in trimethylated H3-K4 upon transcription activation is not due to the decrease in methylated H3-K9 detected by cross-reactive antibody.

To further investigate the apparent decreases in di- and trimethylated H3-K4 as a function of AR occupancy, we monitored both methylation states at various time points after DHT addition. Interestingly, the decreases in di- and trimethylated H3-K4 were rapid (within 15 min) and sustained (up to 24 h, data not shown) at both the PSA enhancer and promoter (Fig. 2). The data suggest that histone replacement and dilution of histone methylation by replication are unlikely

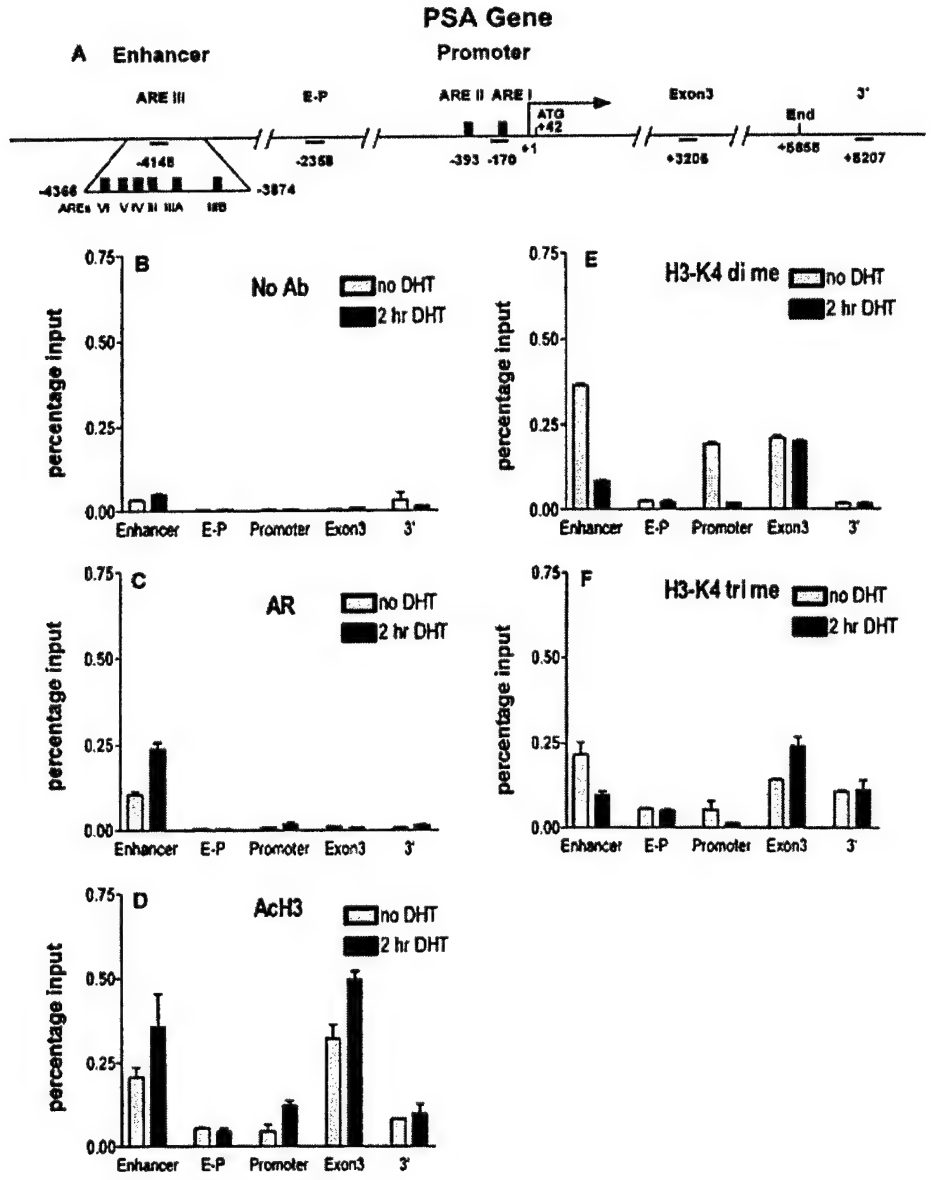

Figure 1. ChIP analysis of AR occupancy and histone H3 modifications at the PSA gene. (**A**) Schematic representation of the PSA gene indicating five target loci (horizontal bars). Vertical bars represent approximate locations of AREs. E-P is a region between the enhancer and promoter without any known function. LNCaP cells were incubated in 5% charcoal-stripped FBS media with either vehicle (EtOH) or DHT (10 nM) for 2 h and analyzed as described in Materials and Methods as follows: (**B**) no antibody control, (**C**) AR occupancy, (**D**) acetylated H3 at lysines 9 and 14, (**E**) dimethylated H3 at lysine 4 and (**F**) trimethylated H3 at lysine 4. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of five independent experiments.

due to the rapid kinetics. To address whether the observed decreases in di- and trimethylated H3-K4 occurred locally at specific loci, we isolated total histones after either 15 min or 4 h of incubation with native ligand and immunoblotted for changes in global H3-K4 methylation. No significant changes in acetylated H3, di- and trimethylated H3-K4 and unmodified H3 were observed after short or prolonged treatment with DHT (Fig. 3). The data suggest that the apparent decreases in di- and trimethylated H3-K4, as well as increase in acetylated H3, are not global effects conferred by DHT treatment but rather are locally regulated events at specific DHT-dependent loci.

Inhibition of AR activity by AR antagonist reverse di- and trimethylation alterations of H3-K4

We assessed whether AR regulates the apparent decreases in di- and trimethylated H3-K4 and, importantly, whether both modifications are reversible. To this end, we quantitated H3-K4 methylation at the enhancer after treating LNCaP cells briefly (30 min) with DHT and then inhibiting AR activity by

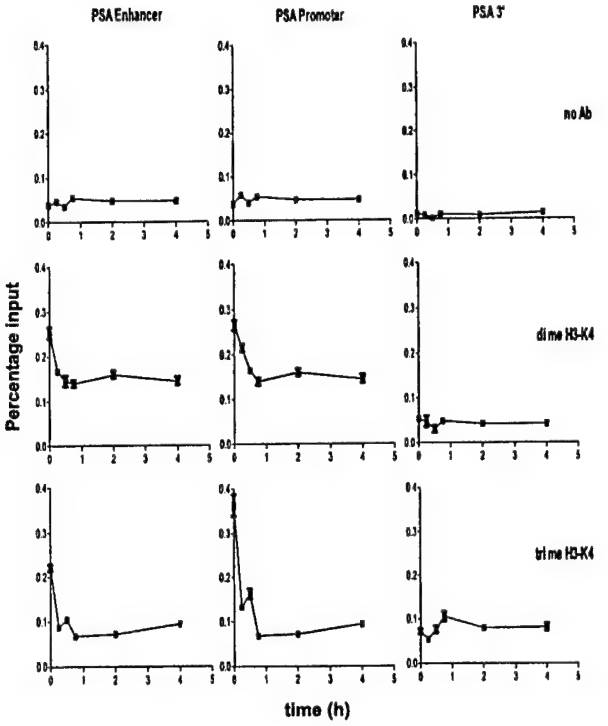

Figure 2. Kinetic analysis of di- and trimethylated H3-K4 at the PSA enhancer, promoter and a 3′ region. LNCaP cells were incubated with either vehicle (representing time 0) or DHT (10 nM) for the indicated times and analyzed using quantitative PCR for no antibody control (top row), dimethylated H3-K4 (middle row) and trimethylated H3-K4 (bottom row) at the PSA enhancer (left column), promoter (middle column) and 3′ region (right column). Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of three independent experiments.

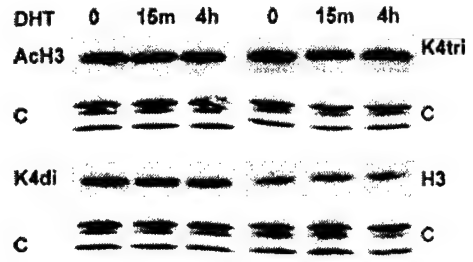

Figure 3. Immunoblot of total histones isolated from LNCaP cells after DHT treatment. Total histones were isolated from LNCaP cells (5×10^6 cells/150 mm dish) after incubation with either vehicle (representing time 0) or DHT (10 nM) for 15 min and 4 h. Total histones were immunoblotted for acetylated H3, dimethylated H3-K4, trimethylated H3-K4 and unmodified H3. C, corresponding Coomassie-stained core histones represent the loading and are shown below each immunoblot. Results shown are representative of three independent experiments.

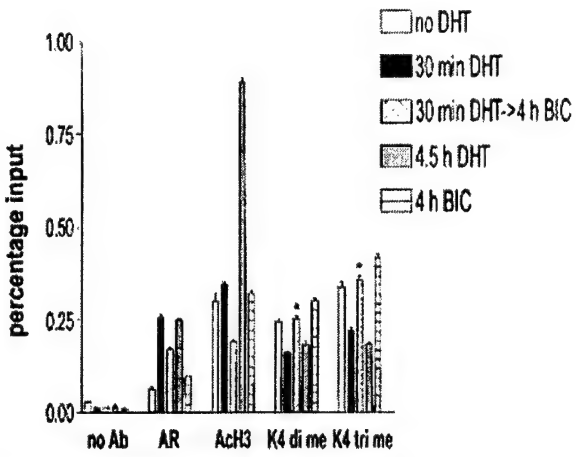

Figure 4. Bicalutamide antagonizes the effect of DHT at the PSA enhancer. LNCaP cells were treated with DHT (10 nM) for 30 min, washed with PBS and treated with bicalutamide (10 μM) for 4 h. ChIP DNA were analyzed as described in Materials and Methods for no antibody control and antibodies against AR, acetylated H3, dimethylated H3-K4 and trimethylated H3-K4. Controls include vehicle, 30 min, 4.5 h DHT and 4 h bicalutamide treatments. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. *Two-tailed *P*-values (*t*-test) of comparisons between the 30 min DHT treatment and bicalutamide reversal values are 0.0012 for dimethylated H3-K4 and 0.0084 for trimethylated H3-K4. Results shown are representative of three independent experiments.

replacing DHT with a specific AR antagonist, bicalutamide. The bicalutamide treatment alone increased AR occupancy by 2-fold but decreased DHT-mediated AR occupancy (Fig. 4), which is consistent with recent evidence that the antagonist functions by binding to AR to induce receptor binding to AREs and recruitment of corepressors such as SMRT (22). Strikingly, the AR-mediated decreases in di- and trimethylated H3-K4 were completely reversed to transcriptionally inactive levels by the bicalutamide treatment. The data indicate that the apparent decreases in di- and trimethylated H3-K4 are regulated in the AR-mediated transcription and are reversible. Furthermore, since the bicalutamide treatment alone can induce AR occupancy but does not decrease the H3-K4 methylation levels, it seems unlikely that either AR or corepressors bind to the methylation signals to mask epitopes in the ChIP assay. However, it is entirely possible that methylated H3-K4 serves as a recognition motif for chromatin-associated proteins that positively regulate transcription such as HATs and coactivators. In this view, AR-mediated transcription or AR itself recruits positive regulators to the transcriptional control regions (i.e. the enhancer and promoter) and bind to di- and/or trimethylated H3-K4 signals. The binding of bulky proteins, or protein complexes, could result in masking of epitopes in ChIP analysis, thereby causing apparent decreases in di- and trimethylated H3-K4.

It has been suggested that one of the possible mechanisms of histone methylation turnover is the proteolytic cleavage of histone tails, specifically between lysines 4 and 9 of histone H3 (23). The proteolysis of H3 tails addresses the active elimination of methylation prior to the histone replacement during DNA replication and, notably, without the elimination of acetylation at lysines 9 and 14. However, this mechanism may not account for our data, which illustrate a dynamic regulation of H3-K4 methylation. An alternate mechanism,

consistent with the active removal of methylation, is the replication-independent exchange of histone H3 with a variant H3.3 (24). In *Tetrahymena*, it was suggested that H3.3 replaces H3 during transcription since H3.3 is only present in the macronucleus where transcription is active (25). Although we cannot rule out this possibility, our data seem to indicate that with the decrease in methylation there is a continued accumulation of acetylation, which may be inconsistent with the mechanism of replacing modified H3 with unmodified H3.3. Still, it may be possible that acetylation and methylation occur on discrete nucleosomes, and only the histones that have been methylated are replaced with the variant.

The active and controlled reversibility of H3-K4 methylation is consistent with the most direct mechanism, which is the enzymatic action of a demethylase. Histone demethylase activity was first reported in 1973 (26), but the enzyme responsible for the activity has never been identified. More recently, it was hypothesized that Elp3, the yeast HAT and elongation factor, is a candidate for the histone demethylase based on its sequence similarity to enzymes that use *S*-adenosyl-L-methionine (SAM) in oxidative reactions (27).

Distinct H3-K4 methylation pattern in the coding region versus transcriptional control regions of the PSA gene

It was reported that dimethylated H3-K4 is present in both the promoter and coding regions of genes in yeast, but with a bias towards the coding regions. Moreover, trimethylated H3-K4 occurs in the 5′ portion of coding regions, which may indicate that trimethylation denotes an early phase of elongation (9,28). Our data suggest that, in human, transcriptional control regions such as the PSA enhancer and promoter are enriched in both di- and trimethylated H3-K4, which can be dynamically regulated. Also, there was an indication that trimethylated H3-K4 in the coding region is regulated (Fig. 1F). To investigate further the role of H3-K4 methylation in the PSA coding region, we quantitated H3-K4 methylation levels at PSA exon 3 over various time periods after induction by DHT. Remarkably, dramatic and significant increases in both di- and trimethylated H3-K4 were observed after a 4 h lag period (Fig. 5A). The qualitative, quantitative and temporal differences in H3-K4 methylation at the coding region versus transcriptional control regions indicate that di- and trimethylated H3-K4 serve distinct roles at those loci. It may be possible that H3-K4 methylation at the transcriptional control regions plays a role in transcription initiation, whereas at the coding region H3-K4 methylation is involved in more downstream processes such as transcription elongation or RNA processing. In yeast, it was shown that Set1, an H3-K4 methylase, is recruited by the RNAP II elongation machinery to the 5′ portion of active coding regions, and the pattern of trimethylated H3-K4 strongly correlates with Set1 occupancy (28). We examined whether trimethylated H3-K4 at the coding region correlates with MLL (the human homolog of Set1) occupancy, and observed that MLL did not locate to either exon 3 or the other loci (data not shown). Together, the data suggest that trimethylated H3-K4 is not limited to the 5′ portion of coding regions, and the accumulation of trimethylated H3-K4 is not due to the recruitment of MLL at exon 3.

To address whether there is a correlation between H3-K4 methylation and PSA mRNA expression, we quantitated both

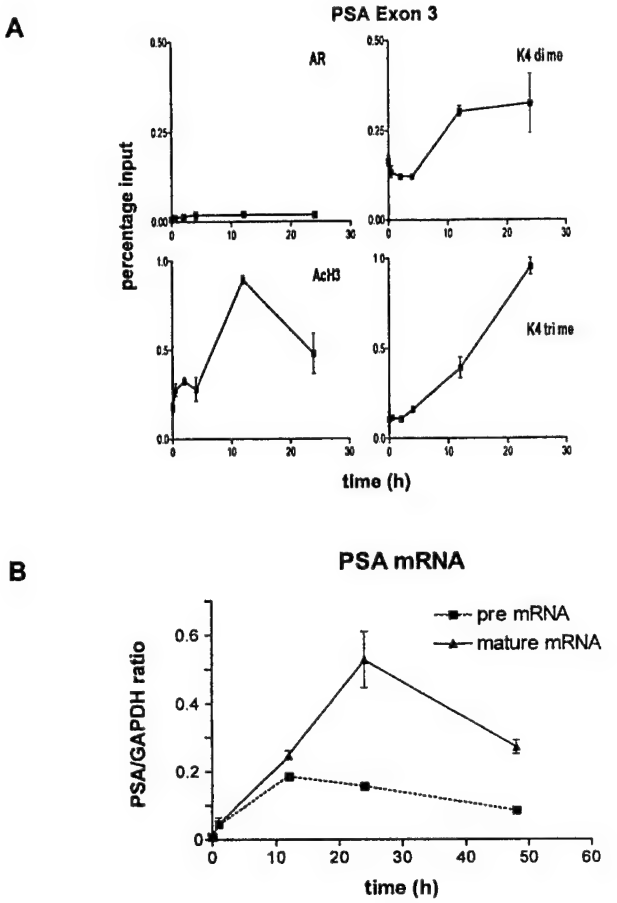

Figure 5. Exon 3 H3-K4 methylation and PSA mRNA expression after prolonged exposure to DHT. (**A**) LNCaP cells were treated with DHT (10 nM) over a 24 h period and analyzed after ChIP of the exon 3 locus using antibodies against AR, acetylated H3, dimethylated H3-K4 or trimethylated H3-K4, as indicated. (**B**) LNCaP cells were treated with DHT over a 48 h period, and pre- and mature mRNA were quantitated by real-time RT–PCR as described previously (15), with forward primers designed in intron 3 and exon 3, respectively, and the reverse primer and probe specific for exon 4. Values are presented as percentage input and error bars represent the standard error of the mean of triplicate determinations using quantitative real-time PCR. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed. Results shown are representative of three independent experiments.

pre- and mature mRNA levels by quantitative real time RT–PCR. The pre-mRNA level reached a plateau at 12 h after ligand induction, whereas the mature mRNA level reached a plateau at 24 h after induction by DHT (Fig. 5B). The increased PSA mRNA production up to 24 h was similar to the kinetics previously reported by others (29). Although the potential positive correlation needs to be investigated further, there seemed to be qualitative and quantitative similarities between the pre-mRNA level and H3-K4 dimethylation and between the mature mRNA level and H3-K4 trimethylation. This raises an interesting possibility that di- and trimethylated H3-K4 serve discrete functions, with trimethylated H3-K4 being associated with more downstream events, such as RNA processing, than dimethylated H3-K4.

Our data clearly demonstrate that the H3-K4 methylation observed in human is different to that in yeast. Thus, the process of marking the coding region with histone methylation signal may be an important mechanism that humans evolved to allow efficient gene expression after a prolonged environmental stimulus. This is also the first demonstration that trimethylated H3-K4 is associated with inactive transcription. Although the exact mechanism of gene regulation conferred by the various covalent modifications is still unclear, it is becoming increasingly evident that understanding the effects of specific modifications such as methylation of histone H3 will be critical to the understanding of the steroid hormone regulation of gene expression.

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Androgen Receptor Signaling: Mechanism of Interleukin-6 Inhibition

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ABSTRACT

Nonsteroidal signaling via the androgen receptor (AR) plays an important role in hormone-refractory prostate cancer. Previously, we have reported that the pleiotropic cytokine, interleukin (IL)-6, inhibited dihydrotestosterone-mediated expression of prostate-specific antigen in LNCaP cells (Jia *et al.*, Mol Can Res 2003;1:385–92). In the present study, we explored the mechanisms involved in this inhibition and considered possible effects on AR nuclear translocation, recruitment of transcription cofactors, and the signaling pathways that may mediate this inhibitory effect. IL-6 neither induced nuclear localization of the AR nor inhibited dihydrotestosterone-induced nuclear translocation of the receptor. IL-6 did not affect AR or p160 coactivator recruitment to the transcription initiation complex on the prostate-specific antigen enhancer and promoter. Moreover, it did not lead to the recruitment of the corepressor silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) or histone deacetylase 1 (HDAC1) at the same sites. IL-6 did, however, prevent the recruitment of the secondary coactivator, p300, to the complex and partially inhibited histone H3 acetylation at the same loci. Furthermore, inhibition by IL-6 was not mediated by the mitogen-activated protein kinase or the Akt pathways and was partially abrogated by signal transducers and activators of transcription-3 knock-down using small interfering RNA. Our results show that IL-6 modulates androgen action through the differential recruitment of cofactors to target genes. These findings may account for the pleiotropic actions of IL-6 in malignant prostate cells.

INTRODUCTION

The only effective systematic treatment for prostate cancer is androgen ablation (1). Although the majority of prostate tumors regress after androgen ablation, disease progression inevitably recurs. Recent evidence suggests that growth of treatment-resistant tumors still depends on maintenance of androgen receptor (AR) signaling pathways (2–4). One mechanism proposed for the maintenance of AR signaling in treatment-resistant prostate tumors is nonsteroidal activation of the AR by cytokines [such as interleukin (IL)-6] and growth factors (such as epidermal growth factor) involving Ras/mitogen-activated protein kinases (MAPKs), Janus-activated kinase (JAK)/signal transducers and activators of transcription (STAT), and/or phosphatidylinositol 3′-kinase/Akt (5–12).

IL-6 is a pleiotropic cytokine produced by a number of cell types and is involved in the regulation of diverse physiological and pathological processes including hematopoiesis and inflammatory responses in target tissues. In prostate cancer, elevated IL-6 serum concentrations are associated with higher prostate-specific antigen (PSA) levels, tumor metastasis, and cancer morbidity (13). The LNCaP cell line, one of the most commonly used androgen-dependent prostate cancer cell models, expresses high-affinity receptors for IL-6 but does not secrete large amounts of IL-6 (14–17). The reported effects of exogenous IL-6 on this cell line are conflicting, with some studies showing growth stimulation (18, 19), and others showing growth inhibition (15, 20). It was recently proposed that binding of IL-6 to its receptor in LNCaP cells results in modulation of three major downstream signaling pathways, Akt, MAPK, and STAT3 (11). Whereas AR transactivation activity was enhanced by the STAT3 or MAPK pathways, activity was suppressed via the Akt pathway in that study. In other studies, IL-6 increased phosphorylation of MAPK and activated the AR possibly via phosphorylation at one of several putative phosphorylation sites (7, 8). The constitutive activation of the Ras/MAPK signaling pathway potentially reduces the androgen requirement of LNCaP cells for growth, PSA expression, and tumorigenicity (21, 22). The involvement of STAT3 after treatment of LNCaP cells with IL-6 is a result of JAK activation (7). STAT3 is a transcription factor that resides in the cytoplasm and most likely has been associated with oncogenesis because of its involvement in the regulation of cell cycle progression and apoptosis (23). A direct interaction between STAT3 and the AR has been proposed as a mechanism by which the JAK/STAT pathway modulates AR activity (7).

In our previous studies, using the endogenous chromatin-integrated PSA gene as a reporter of AR activity, we found that IL-6 inhibited dihydrotestosterone (DHT)-stimulated expression of PSA in LNCaP cells (24). To better understand the mechanism(s) of the IL-6 inhibitory action in LNCaP cells, in the present study we examined the nuclear translocation of the AR and coactivator/corepressor occupancy at different loci of the PSA gene and identified the key nonsteroidal signaling pathways modulating AR activity.

MATERIALS AND METHODS

Cell Culture and Materials. Human prostate cancer LNCaP cells obtained from the American Type Culture Collection (ATCC CRL-1740; Manassas, VA) were maintained in RPMI 1640 supplemented with 5% (v/v) fetal bovine serum and used between passages 25 and 50. DHT and trichostatin A (TSA) were purchased from Sigma Chemical Co. (St. Louis, MO). Recombinant human IL-6 was obtained from R&D Systems, Inc. (Minneapolis, MN). Bicalutamide was obtained from ICI Pharmaceuticals (predecessor of AstraZeneca, London, United Kingdom). Antibodies were anti-AR (N20), anti-SMRT, and anti-actin from Santa Cruz Biotechnology (Santa Cruz, CA); anti-AcH3, anti-p300, anti-HDAC1, and anti-p21 from Upstate Biotechnology, Inc. (Lake Placid, NY); anti-p44/42 MAPK, anti-phospho-p44/42 MAPK, anti-STAT3, anti-phospho-STAT3 (Tyr^{705}), anti-Akt, and anti-phospho-Akt (Ser^{473}) from Cell Signaling Technology, Inc. (Beverly, MA); and anti-PSA from DAKO Corp. (Carpinteria, CA). Anti-AR antibody (U402; sheep polyclonal antibody) was made in our laboratory. U0126 and LY294002 were purchased from Calbiochem-Novabiochem Corp. (San Diego, CA). AG490 was obtained from Promega (Madison, WI). The chimeric AR-green fluorescent protein (GFP) plasmid (kindly provided by Dr Marco Marcelli; Baylor College of Medicine, Houston, TX) was generated by inserting the pCMV-AR vector into the multiple cloning site of the pEGFP-C1 (CLONTECH, Palo Alto, CA) using a *Kpn*I site located 5′ of the ATG start codon and a *Bam*HI site located at the 3′ end of the AR so that the GFP is located 5′ of the AR. The pEGFP-C1 encodes a red-shifted variant of wild-type GFP, which contains a double amino acid substitution of Phe^{64} to Leu and Ser^{65} to Thr.

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Note: W. D. Tilley and G. A. Coetzee codirected this study.

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Immunofluorescence. LNCaP cells (4×10^4 cells/well) were cultured into 8-well chamber slides (Nuclon Lab-Tek II Chamber slide; RS Glass Slide, Naperville, IL) in 600 μl of phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum (CSS) and allowed to grow in steroid-free conditions for 3–5 days. The media were removed and replaced with 600 μl of the same media containing DHT (0–10 nM), IL-6 (10 ng/ml), or IL-6 and DHT in combination. After a 4-h treatment, cells were fixed for 10 min in 4% paraformaldehyde followed by fixation in methanol (−20°C) for 3 min and 1 min in acetone (−20°C) to permeabilize cells. To detect the endogenous AR, cells were incubated overnight at 4°C with affinity-purified sheep polyclonal antibody (U402) to the NH_2-terminal sequence of AR (1:100 dilution) followed by incubation for 1 h with donkey antisheep immunoglobulins conjugated with Alexa 488 in the dark at room temperature. Negative controls included incubation with no primary antibody and blocking peptide. PC3 cells known to be negative for AR were used as a negative control. Specificity of the AR U402 sheep antibody was also confirmed by Western blotting in the presence and absence of blocking peptide. The slides were mounted with fluorescent mounting medium and allowed to air dry overnight in the dark before confocal microscopy.

AR-GFP Transfection Studies. LNCaP cells transfected with the AR-GFP plasmid were investigated. LNCaP cells (7×10^4 cells/well) were cultured into 8-well chamber slides (Nuclon Lab-Tek II Chamber slide; RS Glass Slide), allowed to adhere for 48 h, and then transfected with 2 μg/well AR-GFP plasmid using LipofectAMINE 2000 reagent (Invitrogen Corp., Carlsbad, CA) as per the manufacturer's instructions, with minor modifications. Transfection media were removed, and media were replaced after 20 h with 600 μl of phenol red-free RPMI 1640 containing 5% CSS with vehicle (ethanol), DHT (1 nM-10 nM), IL-6 (10 ng/ml), or IL-6 in combination with DHT. After a 24-h treatment, the media with fresh cytokine and/or ligand were replenished, and the cells incubated for an additional 4 h before fixation and confocal microscopy.

Confocal Microscopy and Imaging Analysis. The images of endogenous AR and transfected AR-GFP were produced using the Bio-Rad Radiance 2100 confocal microscope (Bio-Rad Microscience Ltd., Hemel Hempstead, United Kingdom) equipped with three lasers [argon ion 488 nm (14 mW), Green HeNe 543 nm (1.5 mW), and Red Diode 637 nm (5 mW) outputs] and an Olympus IX70 inverted microscope. The ×40 water objective was used, and images were collected at zoom 2.00×. The Alexa 488 or GFP was excited with Ar 488 nm laser line, and the emission was viewed through a HQ515/30 nm narrow band barrier filter in photomultiplier 1. The density of endogenous AR in the cytoplasm of untreated and treated cells was measured using the LaserPix software program for the Microsoft Windows (Bio-Rad Microscience Ltd.). The expression of endogenous AR was examined in up to 60 cells in three separate experiments. The one-way ANOVA test and the Dunnet *post hoc* test were used to determine statistical significance between control and treatment groups. All analyses were performed using SPSS 11.0 for Windows Software (SPSS Inc., Chicago, IL). Statistical significance was accepted at $P < 0.05$.

Plasmid Transfection and Luciferase Detection. LNCaP cells (5×10^4 cells/well) were plated in 96-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 3 days. Cells were then transfected with an AR-responsive reporter, PSA-luc [100 ng/well; pGL3_PSA540-luc; provided by Bristol-Myers Squibb, (Princeton, NJ); Ref. 24], and pCMV-p300 [50 ng/well; provided by Dr. T-P. Yao (Duke University, Durham, NC)] or pCAT-basic (50 ng/well; Promega), a negative control, using LipofectAMINE 2000 (Invitrogen Corp.) according to the manufacturer's protocol. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS with DHT and/or IL-6 as indicated for 30 h. Luciferase assays were conducted as described previously (24). The results are given as fold activation and represent means ± SD of quadruplicate wells.

Small Interfering RNA (siRNA) Transfection. LNCaP cells (3×10^5 cells/well) were plated in 6-well plates and grown in phenol red-free RPMI 1640 containing 5% CSS for 3 days. Cells were transfected with STAT3 siRNA or nonspecific siRNA (Dharmacon Inc., Lafayette, CO) at final concentration of 100 nM using Oligofectamine reagent (Invitrogen Corp.) according to the manufacturer's instructions. After transfection, cells were grown in phenol red-free RPMI 1640 containing 5% CSS for 48 h and then treated with DHT and/or IL-6 as indicated for 18 h. Total RNA extraction and protein extraction were conducted respectively for further assessment.

Real-Time Reverse Transcription-PCR. Total cellular RNA was prepared and treated with RNase-free DNase I using SV Total RNA Isolation System (Promega). A two-step reverse transcription-PCR method was used using the TaqMan Gold reverse transcription-PCR kit (Applied Biosystems, Branchburg, NJ). The primers and probes were described previously (24). Triplicate PCR reactions were routinely conducted. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expression was analyzed for each sample in parallel wells. The results are represented as PSA/GAPDH mRNA ratio.

Immunoblotting. After treatment with DHT and/or IL-6 as indicated, cells were harvested in 100 μl of radioimmunoprecipitation assay buffer [10 μM sodium phosphate (pH 7.2), 2 mM EDTA (pH 8.0), 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% IGEPAL CA-630, 1% sodium deoxycholate, and 0.2 mM Na_3VO_4] that contained a mixture of mammalian protease inhibitors (Sigma Chemical Co.). Equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and probed with different antibodies as indicated. Horseradish peroxidase-conjugated antirabbit IgG antibody or horseradish peroxidase-conjugated antigoat IgG antibody (Santa Cruz Biotechnology) was used as the secondary antibody. Detection was performed using the Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology) according to the manufacturer's protocol. Chemiluminescence was analyzed using a Fluor-S Multi-Imager MAX instrument (Bio-Rad, Hercules, CA).

Chromatin Immunoprecipitation Assay. LNCaP cells (7×10^6 cells/150-mm dish) were cultured in phenol red-free RPMI 1640 supplemented with 5% CSS for 3 days. Cells were treated with DHT and/or IL-6 for various times as indicated, and chromatin immunoprecipitation assays were conducted as described previously (24). Briefly, cells were cross-linked at room temperature for 10 min by using 1% formaldehyde. After sonication, the resulting soluble chromatin was diluted 1:10 with dilution buffer and immunoprecipitated by incubation with the indicated specific antibodies overnight at 4°C with rotation. The following day, chromatin-antibody complexes were isolated from solution by incubation with protein G-Sepharose beads (Amersham Pharmacia Biotech) for 1 h at 4°C with rotation. The Sepharose-bound immune complexes were washed as described previously. The immune complexes were eluted from beads with an elution buffer (1% SDS and 0.1 M NaHCO3) followed by DNA extraction. DNA samples from chromatin immunoprecipitation preparations were analyzed by real-time PCR using an iCycler optical system (Bio-Rad) and AmpliTaq Gold PCR master mix (Applied Biosystems). The primers and probes were as follows: enhancer forward (5′-GCCTGGATCTGAGAGAGATATCATC-3′) and reverse (5′-ACACCTTTTTTTTTCTGGATTGTTG-3′); E-P forward (5′-CAGTGGCCATGAGTTTTGTTTG-3′) and reverse (5′-AACCAATCCAACTGCATTATACACA-3′); promoter forward (5′-CCTAGATGAAGTCTCCATGAGCTACA-3′) and reverse (5′-GGGAGGGAGAGCTAGCACTTG-3′); enhancer probe (5′-6-FAM-TGCAAGGATGCCTGCTTTACAAACATCC-BHQ-1-3′); E-P probe (5′-6-FAM-CCCAACGCAACTTAACCTAACAAG-BHQ-1-3′); and promoter probe [5′-6-FAM-CAATTACTAGATCACCCTGGATGCACCAGG-BHQ-1-3′ (Biosearch Technologies, Novata, CA)]. Triplicate PCR reactions for each sample were conducted. The results are given as a percentage of input and represent mean values ± SD of triplicate determinations.

RESULTS

IL-6 Inhibits DHT-Mediated PSA Expression. We have reported previously that IL-6 inhibited DHT-mediated PSA expression at 10 nM DHT concentrations (24). The inhibition was also apparent at lower DHT concentrations (Fig. 1), indicating a general inhibitory mechanism across all tested concentrations of DHT, including expected castrate levels of the androgen.

IL-6 Does Not Affect the Distribution and DHT-Induced Nuclear Translocation of the AR. To investigate the possible mechanistic interactions between IL-6 and AR activity, initially we evaluated the effect of IL-6 on AR subcellular localization using confocal microscopy. Although endogenous AR was observed in both the cytoplasm and nuclei in the absence of ligand, the majority was nuclear (Fig. 2*A*). Treatment with either 1 or 10 nM DHT decreased

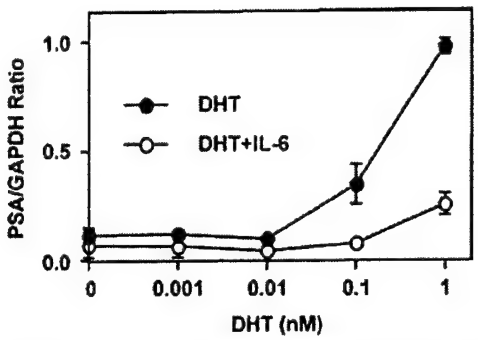

Fig. 1. Inhibitory effect of interleukin-6 on dihydrotestosterone-mediated prostate-specific antigen (PSA) expression in LNCaP cells. LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with different concentrations of dihydrotestosterone as indicated with or without interleukin-6 (10 ng/ml) for 18 h. PSA and GAPDH mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as the means ± SD of triplicate real-time PCR assays.

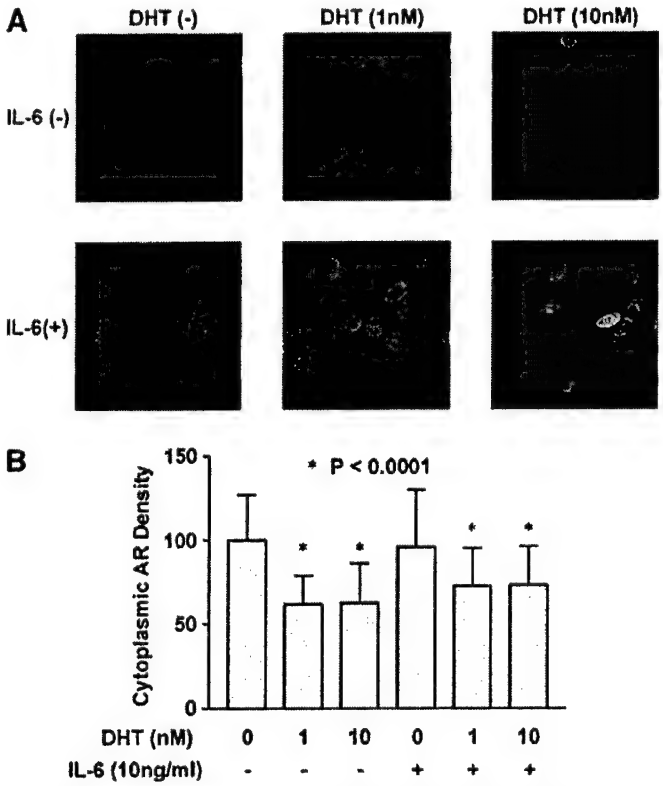

Fig. 2. Endogenous androgen receptor (AR) distribution in LNCaP cells after dihydrotestosterone (DHT) and/or interleukin-6 treatment. *A*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with interleukin-6 (10 ng/ml) either independently or in combination with 1–10 nM DHT for 4 h. Immunostaining with antibody against AR (U402) was performed to examine the distribution of endogenous AR. *B*, LNCaP cells were scanned using confocal microscopy. Cytoplasmic AR expression values are shown as a percentage of untreated control. ▨, mean values of three independent experiments; *error bars*, SD. *P* was calculated using one-way ANOVA test and the Dunnet *post hoc* test to compare DHT-mediated cytoplasmic densities with the corresponding non-DHT-treated controls.

cytoplasmic AR levels to about 60% of untreated levels ($P < 0.0001$; Fig. 2*B*). The cellular distribution of AR in cells treated with IL-6 alone was not different from that seen in untreated controls. AR distribution in cells treated with DHT and IL-6 in combination was similar to that in cells treated with DHT alone (Fig. 2, *A* and *B*). No AR immunostaining was observed in the negative controls. In parallel experiments using a transiently expressed AR-GFP, the AR was both cytoplasmic and nuclear in untreated controls and in cells treated with IL-6 (10 ng/ml) alone. DHT (1 nM)-induced nuclear localization of the fusion protein was not prevented by combined treatment with IL-6 and DHT (Fig. 3).

IL-6 Interferes with DHT-Mediated Transient p300 Recruitment. In our experience, IL-6 consistently inhibits endogenous PSA gene expression in LNCaP cells (25). To understand the precise mechanism of this IL-6 inhibitory activity, we analyzed the AR occupancy, histone H3 acetylation, and cofactor recruitment on the PSA enhancer and promoter and a sequence between them (E-P; Fig. 4*A*) during the first 4 h of treatment with DHT or DHT + IL-6. IL-6 had little effect on DHT-mediated AR occupancy, which occurs predominantly on the enhancer, but significantly inhibited histone H3 acetylation, consistent with our previous findings (Ref. 25; Fig. 4*B*). GRIP1, a member of the p160 family of coactivators, functions as a molecular adapter. Recruitment of GRIP1 to the PSA enhancer and promoter after treatment with DHT was not affected by IL-6 treatment. In contrast, the DHT-mediated transient recruitment of p300 on both the promoter and the enhancer was totally inhibited by IL-6 (Fig. 4*B*). The transient nature of p300 recruitment by DHT treatment was a constant finding in three independent experiments and was also reported by other researchers (26, 27). Neither AR, cofactor occupancy, nor histone H3 acetylation was observed in the E-P region. Neither silencing mediator of retinoic acid and thyroid hormone receptor (SMRT) nor histone deacetylase 1 (HDAC1) was recruited to any of the three sites (Fig. 4*B*). In similar experiments, SRC1, RAC3, TAF II, NcoR, HDAC2, and HDAC3 also yielded negative results (data not shown).

We confirmed that SMRT and HDAC1 could be recruited to the PSA promoter and enhancer by treating the cells with the AR antagonist bicalutamide. Bicalutamide recruited AR to both the promoter and enhancer regions of the PSA gene, although the levels of AR occupancy are much lower than those obtained after DHT treatment (Fig. 5*A*). SMRT and HDAC1 were recruited to both loci under these conditions (Fig. 5*A*), indicating that the lack of SMRT/HDAC1 recruitment to these sites after stimulation with IL-6 was not due to failure of the chromatin immunoprecipitation assay. To further demonstrate that IL-6 inhibition of PSA expression is not related to HDAC recruitment, we examined DHT-mediated PSA expression after IL-6 treatment with or without TSA, a HDAC inhibitor. Although TSA increased DHT-mediated PSA expression by about 20% (data not

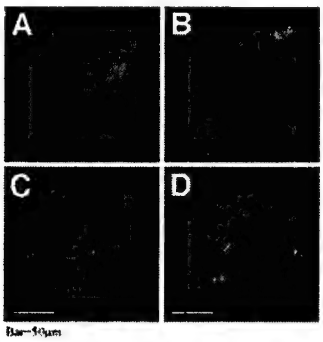

Fig. 3. Localization of expressed androgen receptor-green fluorescent protein in transiently transfected LNCaP cells after interleukin (IL)-6 treatment. LNCaP cells were cultured in 5% fetal bovine serum RPMI 1640 for 48 h. Cells were then transfected with androgen receptor-green fluorescent protein plasmid. After 20 h, the transfection media were removed and replaced with phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum with vehicle (ethanol; *A*), dihydrotestosterone (1 nM; *B*), IL-6 (10 ng/ml; *C*), or IL-6 in combination with dihydrotestosterone (*D*) for 24 h. The media with fresh cytokine and/or ligand were replenished then, and the cells were incubated for an additional 4 h before fixation.

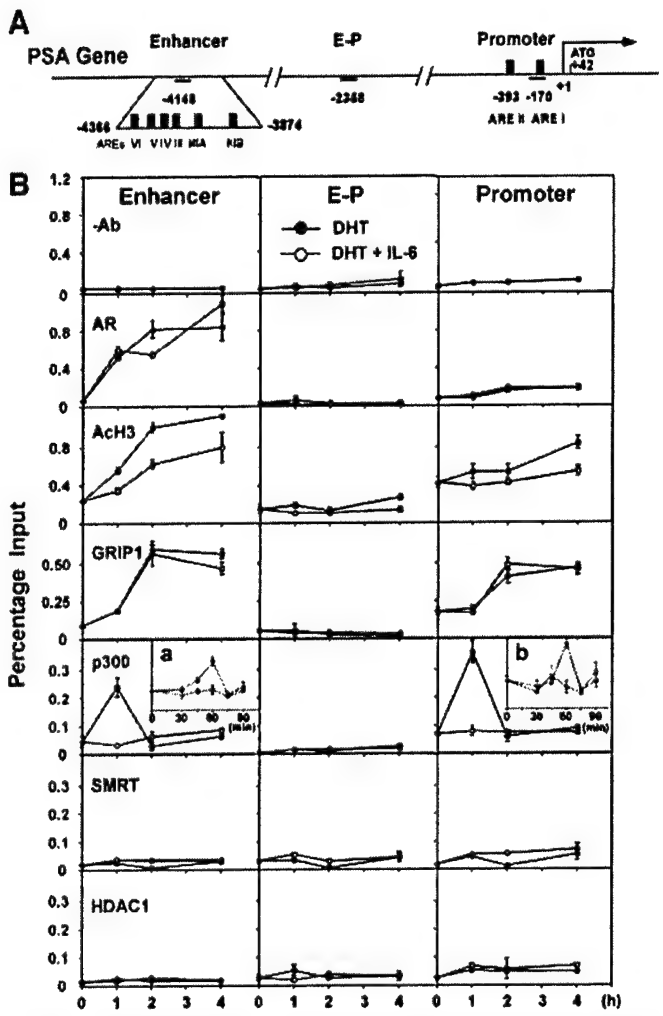

Fig. 4. Influence of interleukin (IL)-6 on dihydrotestosterone (DHT)-mediated transcription complex formation on the prostate-specific antigen gene enhancer and promoter. *A*, schematic representation of the prostate-specific antigen gene promoter and enhancer regions. *Vertical dark bars*, locations of AREs. *Horizontal dark bars*, real-time PCR targeted regions. *E-P* is a region between the enhancer and promoter without any known function. *Arrow*, transcription start site. *B*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with DHT (10 nM) alone or in combination with IL-6 (10 ng/ml) for various times as indicated. Androgen receptor occupancy, histone H3 acetylation, and recruitment of cofactors were examined by chromatin immunoprecipitation analyses. *p300 inset graphs*, in a separate chromatin immunoprecipitation experiment, p300 recruitment on both enhancer (*a*) and promoter (*b*) were analyzed at more time points around the 1 h time point for both the DHT and DHT + IL-6 treatment regimens. Values are presented as percentage input and represent the mean values ± SD of triplicate real-time PCR assays. Input values were obtained from samples treated in the same way as the experimental ones, except that no immunoprecipitation steps were performed.

shown), TSA had no significant effect on the IL-6-induced inhibition (Fig. 5*B*). In contrast, TSA treatment reversed the bicalutamide-induced inhibition of PSA expression by about 2-fold (Fig. 5*C*).

Transient p300 Overexpression Does Not Abrogate IL-6 Inhibition of AR Transactivation Activity. In an attempt to manipulate p300 activity, we cotransfected a mammalian expression vector of p300 along with a PSA promoter/enhancer-driven luciferase reporter into LNCaP cells that were subsequently treated with or without IL-6 and/or DHT (Fig. 6). IL-6 inhibited AR activity by 31%, and although p300 overexpression significantly stimulated the activity by more than 2-fold, IL-6 maintained an inhibition of 46%. This result is consistent with an inhibition of p300 recruitment to the PSA enhancer/promoter. On the other hand, p300 overexpression still resulted in increased luciferase activity even during IL-6 treatment. This finding indicates that the inhibition observed by IL-6 cannot be due solely to inhibition of all p300 functions in this overexpression system. The results from this experiment should be interpreted with caution because we assayed a transient reporter plasmid and not the endogenous gene.

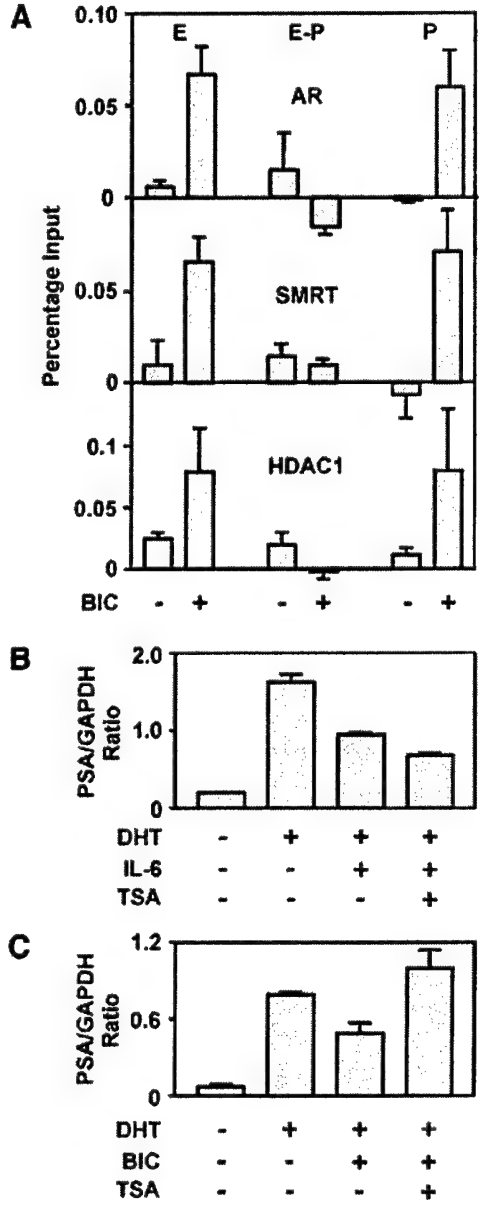

Fig. 5. Bicalutamide and corepressor recruitment. *A*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with bicalutamide (5 μM) for 1 h. Chromatin immunoprecipitation analyses of androgen receptor, SMRT, and HDAC1 on the prostate-specific antigen (PSA) gene promoter and enhancer were performed. Values are presented as described in Fig. 2*A* (except that the matched no antibody value was subtracted from each value). *B*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with different combinations of dihydrotestosterone (10 nM), interleukin-6 (10 ng/ml), and trichostatin A (100 nM) for 18 h. PSA mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as the means ± SD of triplicate real-time PCR assays. *C*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with different combination of dihydrotestosterone (10 nM), interleukin-6 (10 ng/ml), and bicalutamide (5 μM) for 18 h. PSA mRNA levels were measured and analyzed as described in *B*. Results shown are representative of at least two independent experiments.

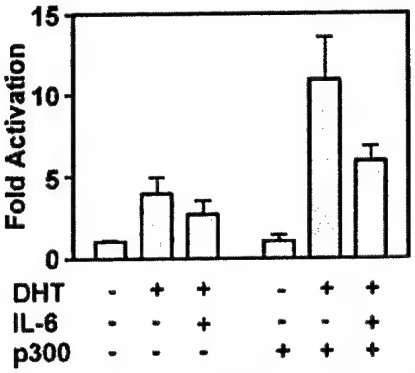

Fig. 6. Effects of p300 overexpression. LNCaP cells were transiently transfected with PSA-luc (100 ng/well) and pCMV-p300 (50 ng/well) or control vector pCAT-basic (50 ng/ml). Cells were then incubated with dihydrotestosterone (10 nM) and/or interleukin-6 (10 ng/ml) for 30 h. Values are presented as the means ± SD of quadruplicate wells.

Inhibiting MAPK or Akt Pathways Does Not Restore IL-6-Induced Inhibition of PSA Expression. We used specific inhibitors to determine the relative contribution of the JAK/STAT3, MAPK, and phosphatidylinositol 3′-kinase/Akt signaling pathways to the IL-6 inhibitory activity on PSA expression (Fig. 7*A*). The efficacy of the inhibitors was evaluated by immunoblot analysis of phosphorylated target proteins. IL-6-induced p44/42 MAPK phosphorylation was totally inhibited by MAPK kinase inhibitor U0126 (Fig. 7*B*), but this did not influence DHT-dependent PSA expression or the inhibitory activity of IL-6 (Fig. 7*D*). Because Akt is constitutively active in LNCaP cells, phosphorylation of Akt was independent of IL-6 stimulation (Fig. 7*C*). In addition, the level of phosphorylated Akt remained unchanged in LNCaP cells cultured in RPMI 1640 without serum for 2 days (data not shown). Inhibition of the constitutively active Akt pathway by LY294002 (Fig. 7*C*) dramatically inhibited DHT-dependent PSA mRNA expression, indicating a significant involvement of this pathway in modulating AR activity. The magnitude of IL-6 inhibitory activity remained unaffected by LY294002 treatment (Fig. 7*E*).

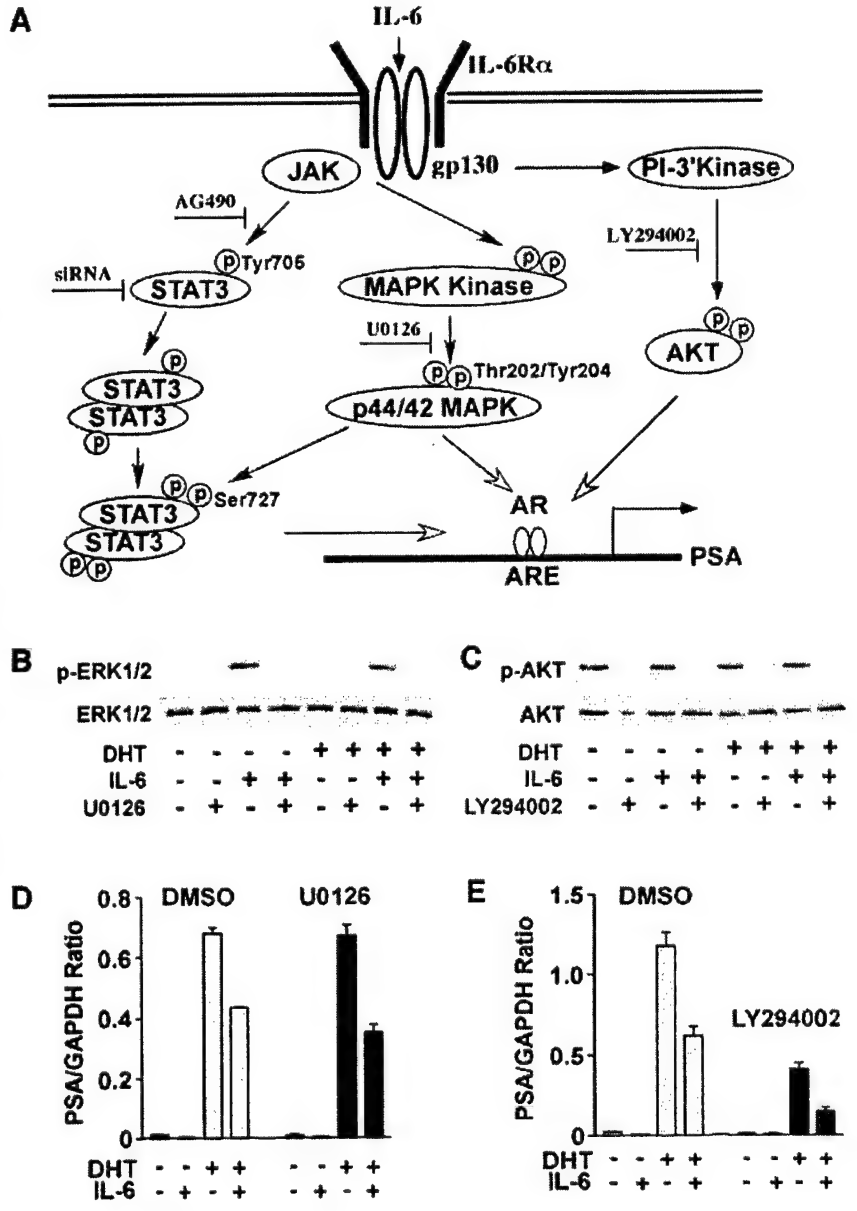

Fig. 7. Prostate-specific antigen (PSA) mRNA expression after the inhibition of the mitogen-activated protein kinase (MAPK) or phosphatidylinositol 3′-kinase/Akt signaling pathway. *A*, proposed model of the contribution of interleukin (IL)-6-induced signal transducers and activators of transcription, MAPK, and phosphatidylinositol 3′-kinase/Akt signal transduction pathways to androgen receptor activity. *B*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with dihydrotestosterone (10 nM) and/or IL-6 (10 ng/ml) for 15 min after a pretreatment period of 1 h with U0126 (10 μM) or DMSO control vehicle. Immunoblots of phosphorylation of extracellular signal-regulated kinase (ERK) 1/2 (p44/42 MAPK) were conducted using anti-phospho-ERK1/2 and anti-ERK1/2 antibodies. *C*, in a separate experiment, LNCaP cells were treated as described in *B* but pretreated with LY294002 (20 μM) or DMSO for 1 h. Western analyses of phosphorylation of Akt were conducted using anti-phospho-Akt and anti-Akt antibodies. *D*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then pretreated with U0126 (10 μM) or DMSO for 1 h before the addition of dihydrotestosterone (10 nM) and/or IL-6 (10 ng/ml) for an additional 18 h. PSA mRNA levels were measured by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as the means ± SD of triplicate real-time PCR assays. *E*, in a separate experiment, LNCaP cells were treated as described in *D* but pretreated with LY294002 (20 μM) or DMSO for 1 h. PSA mRNA levels were measured as described in *D*. Results shown are representative of two independent experiments.

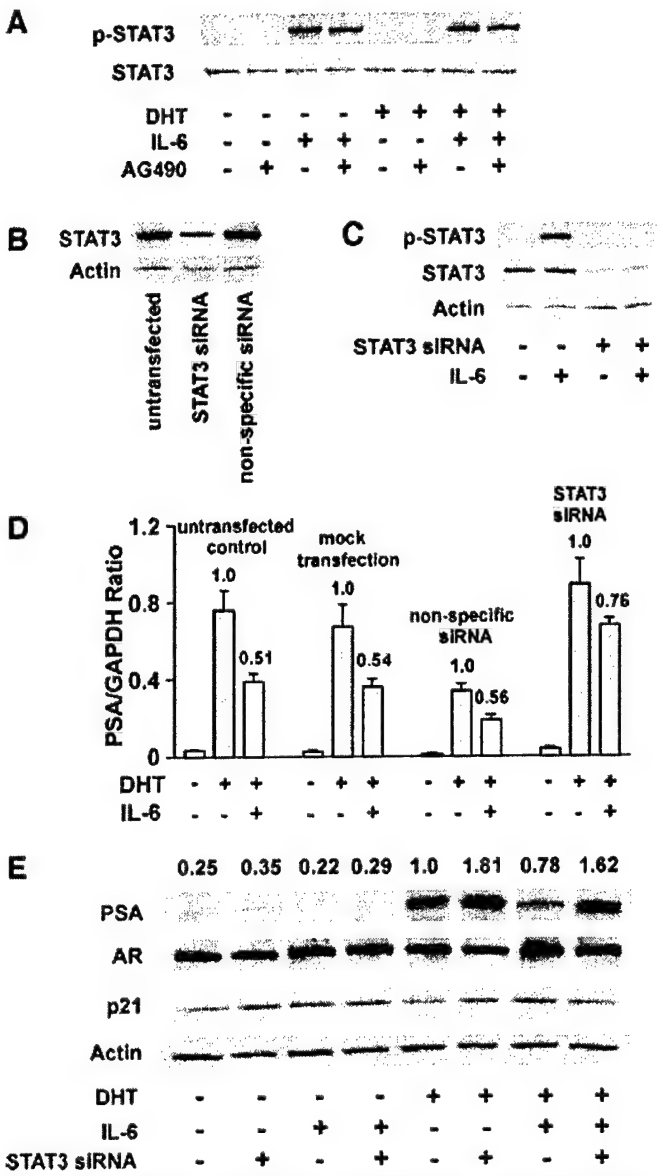

Fig. 8. Up-regulation of prostate-specific antigen (PSA) expression after small interfering RNA (siRNA)-mediated knock-down of signal transducers and activators of transcription-3 (STAT3). *A*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then treated with dihydrotestosterone [DHT (10 nM)] and/or interleukin (IL)-6 (10 ng/ml) for 15 min after pretreatment with AG490 (50 μM) or DMSO for 1 h. Immunoblots of the phosphorylation status of STAT3 were conducted using anti-phospho-STAT3 and anti-STAT3 antibodies. *B*, LNCaP cells were incubated in phenol red-free RPMI 1640 containing 5% charcoal/dextran-stripped fetal bovine serum for 3 days and then transfected with STAT3 siRNA (100 nM) or the same amount of nonspecific siRNA. After 48 h, STAT3 protein levels were measured by immunoblot analysis. The membrane was stripped and reprobed with an anti-actin antibody. *C*, 48 h after STAT3 siRNA or nonspecific siRNA transfection, LNCaP cells were treated with or without IL-6 (10 ng/ml) for 15 min. Phosphorylation of STAT3 was examined by immunoblot analysis using anti-phospho-STAT3 antibody. Total STAT3 was detected by anti-STAT3 antibody. *D*, 48 h after transfection with STAT3 siRNA or nonspecific, scrambled siRNA (untreated and mock-transfected controls are included), LNCaP cells were treated with DHT (10 nM) alone or in combination with IL-6 (10 ng/ml) for another 18 h. PSA mRNA expression levels were examined by real-time reverse transcription-PCR. The PSA expression values are shown as PSA/GAPDH mRNA ratios. Values are presented as the means ± SD of triplicate real-time PCR assays. The *number above the columns* indicates the relative value to DHT-mediated PSA expression value (defined as 1.0). *E*, in a separate experiment, LNCaP cells were transfected with STAT3 siRNA or nonspecific, scrambled siRNA and treated as described in *D*. Immunoblot analysis of endogenous PSA, AR, p21, and actin protein levels was conducted. The *number above each lane* of PSA expression indicates the relative intensity of each band (defined as 1.0) normalized to actin expression. Results shown are representative of three independent experiments.

IL-6 Inhibition of PSA Expression Is Partially Abrogated after Knock-Down of STAT3. Although STAT3 phosphorylation was stimulated by IL-6 treatment (Fig. 8*A*), we were unable to inhibit STAT3 phosphorylation with the putative JAK inhibitor AG490. Increasing the concentration of AG490 to 100 μM (10× the IC_{50}) and extending pretreatment time to 16 h did not inhibit phosphorylation of STAT3 but instead induced cytotoxicity (data not shown). Therefore, we used an alternative approach of knocking-down STAT3 expression with siRNA.

After transfection of LNCaP cells with STAT3 siRNA, total endogenous STAT3 levels were reduced by 70–80% (range of inhibition in three separate experiments as assessed by Western quantitation; Fig. 8*B*). IL-6-induced phosphorylation of STAT3 was also inhibited to a similar extent (about 70%) by STAT3 siRNA transfection (Fig. 8*C*). DHT-mediated and IL-6-inhibited PSA expression levels were reduced by the transfection of scrambled, control siRNA compared with untreated or mock-transfected controls, indicating a general siRNA effect on the cells (Fig. 8*D*). The inhibition by IL-6 remained at about 50% under these conditions. Nevertheless, the specific knock-down of STAT3 increased DHT-mediated and partially abrogated the IL-6 inhibitory effects on PSA mRNA and protein levels (Fig. 8, *D* and *E*, respectively). GAPDH mRNA levels remained essentially unchanged by all of the above-mentioned treatments. In the same experiment, knock-down of STAT3 did not significantly affect endogenous AR and p21 levels (Fig. 8*E*).

DISCUSSION

In prostate cancer, elevated IL-6 levels have been correlated with metastasis, morbidity, and poor prognosis (17, 28, 29). However, it is not clear how IL-6 signaling is involved in prostate cancer progression because previously published data have yielded conflicting results regarding the effects of IL-6 on prostate cancer cell proliferation (15, 18–20). We reported previously that in early- passage LNCaP cells, IL-6 inhibited DHT-stimulated cell growth and PSA gene expression (24). To gain insight into possible mechanisms involved in this inhibition, we considered, in the present study, nuclear translocation of the AR, recruitment of cofactors to the PSA enhancer and promoter, and, finally, which signaling pathway may be involved between the cell surface binding of IL-6 and DHT-mediated PSA expression.

In the present study, we observed persistent nuclear expression of endogenous AR after 3–5 days of culture in steroid-free medium. Similarly, nuclear AR was also detected by Lin *et al.* (30) in studies using LNCaP. However, contrary to the latter report, we did not observe increases in nuclear AR levels after IL-6 treatment. Cell-specific factors are known to alter AR nuclear trafficking (31) and may account for the differences between our observations and those of others.

It is well established that AR activates gene transcription by binding to specific androgen response elements on androgen-regulated target genes and recruiting coactivators in a ligand-dependent manner starting with the p160 family members, followed by p300 complexes (26). p300 recruits additional factors with histone acetyltransferase (HAT) activity, such as p300/CREB-binding protein (CBP)-associated factor, leading to histone acetylation, chromatin modification, and transcription initiation. Here, we found that IL-6 inhibited histone acetylation by interference with the DHT-induced transient p300 recruitment to p160 at the PSA enhancer and promoter. The reason of the transient nature of p300 recruitment is unknown. It might be an artifact of epitope masking, or it could be due to its replacement by other cofactors with additional HAT activities for further maintenance of histone acetylation. In this regard, our results are directly opposite

to that reported previously (32). The reason for this is unknown but might be related to the passage number of LNCaP cells used; we routinely use LNCaP cells at a passage number as low as possible and never beyond 50 passages. We also demonstrated that the mechanism of IL-6 inhibitory activity on PSA expression was different from that of the AR antagonist bicalutamide, which binds AR and recruits corepressors and HDAC (26, 33).

It is interesting to note that although AR recruitment levels to the promoter of the PSA gene were much lower than those to the enhancer, p160 and p300 recruitment to both loci were nearly equivalent. The reason for the lower AR occupancy at the promoter might be related to the fewer response elements at the promoter compared with the enhancer, but the high p160/300 occupancy at the promoter might be the result of bridging activity of the cofactors between the two sites after DNA looping. It is known, for example, that p160 cofactors have more than one AR binding site (34) that might facilitate such bridging.

Emerging evidence indicates that coactivators and corepressors are themselves targets of multiple signal transduction pathways (35). Both p160 cofactors (36) and p300 (37) can be modulated by the action of several kinases. Activation of various intracellular signaling pathways and kinase cascades by membrane receptors modulates coactivator complexes and seems to determine which acetyltransferases, such as p300/CBP or p300/CBP-associated factor, are recruited to a particular coactivator complex in a specific context. Although the transient nature of p300 recruitment after treatment with DHT remains unexplained, a similar observation was made for estrogen receptor-regulated transcription complexes in which the transient recruitment of p300 was replaced by CBP (38).

In a recent report, IL-6 was shown to enhance AR transactivation via either STAT3 or MAPK pathways and suppress AR transactivation via the phosphatidylinositol 3′-kinase/Akt pathway (11). We found, however, that the Akt pathway might be a major constitutive contributor to nonsteroidal AR activation in LNCaP cells. Blocking this pathway significantly reduced DHT-mediated PSA expression without affecting inhibition of IL-6. In our experiments, IL-6 inhibited PSA gene expression, at least in part, through the STAT3 pathway without MAPK involvement, which is consistent with a report that the activation of STAT3 is the main underlying mechanism for IL-6-induced growth inhibition (39). This inhibitory activity did not result from modulation of endogenous AR and seems to be gene specific because we showed that AR and p21 protein levels remained unchanged. We speculate that activation of STAT3 would block the association between p160 and p300 on the PSA gene enhancer and promoter, thereby releasing HAT and preventing histone acetylation. It remains unknown how activation of STAT3 may interact with AR and/or specific cofactors to influence the recruitment of p300 to transcription initiation complexes on the PSA gene promoter and enhancer regions. Because we did not find STAT3 occupancy on either the promoter or enhancer of PSA gene (data not shown), it remains possible that the JAK/STAT3 pathway activated a transcriptional repressor (such as cyclin D1) that inhibits PSA expression. Cyclin D1 can bind the amino-terminal domain (NTD) of the AR independent of its *LXXLL* motif and functions as a corepressor to inhibit ligand-dependent AR activation. Because cyclin D1-mediated repression is dominant over both HAT and HAT recruiting coactivators (40), it has been suggested recently that cyclin D1 binding to the AR may repress ligand-dependent AR activity by directly competing for p300/CBP-associated factor binding (41). Moreover, protein inhibitor of activated STAT3 (PIAS3) may also play a role in this process; PIAS3, originally identified as a specific inhibitor of STAT3, was recently shown to interact with the AR and suppress AR-mediated gene activation (42, 43). It should be noted that the transfection of control, scrambled siRNA resulted in a significant inhibition of PSA mRNA expression (under both DHT-treated and DHT+IL-6-treated conditions). This inhibition could possibly be due to general effects of duplex RNA species on the IFN-mediated activation of JAK/STAT pathways (44). If this is true, our results are consistent with an inhibitory activity of STAT3 on DHT-mediated PSA expression, which was rescued by the specific knock-down of STAT3.

Whereas the pleiotropic effects of IL-6 on the LNCaP phenotype, as reported by different groups, are puzzling, the challenge is to sort out how and by what mechanisms different signaling pathways affect AR-mediated gene expression. A better understanding of the diverse mechanisms involved in the regulation of AR signaling is essential to better control the growth of prostate cancer cells and, in particular, prevent the development of hormone-refractory prostate cancer, which currently is not amenable to alternative treatments. Conceivably, differential regulation of AR activity by multiple signaling pathways, including IL-6, could significantly affect the phenotype of prostate cancer cells and contribute to uncontrolled growth. Molecular detail of nonsteroidal modulation of the AR in the future might lead to cytokine-mediated control of prostate cancer cell growth, especially in a castrate environment.

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PC-3 Cells With Enhanced Androgen Receptor Signaling: A Model for Clonal Selection in Prostate Cancer

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BACKGROUND. Two sublines of the human prostate cancer cell line, PC-3, which is widely used as a model of prostate cancer progression, have been reported: PC-3^{AR-} that do not express androgen receptor (AR), and PC-3^{AR+} that have measurable AR RNA but little protein.
METHODS. We assayed the geneotype, karyotype, AR expression, and physical characteristics of the two PC-3 sublines, and compared their ability to elicit a transactivation response from ectopic AR in the presence and absence of specific AR coregulators.
RESULTS. PC-3^{AR-} and PC-3^{AR+} cells are genotypically and karyotypically similar, but exhibit salient differences in their morphology, growth rate, and expression of AR RNA. Whereas endogenous AR expression in PC-3^{AR+} cells does not result in sufficient protein to confer androgen responsiveness in culture, ectopic AR consistently elicited a much greater transactivation response in PC-3^{AR+} than in PC-3^{AR-} cells, without altered sensitivity to activation by native ligand or AR coregulators including GRIP1, BRCA1, and Zac1. Moreover, phenotypic differences of AR variants implicated in prostate cancer susceptibility and progression were only observed in PC-3^{AR+} cells. Higher levels of known AR coregulator proteins detected in PC-3^{AR+} compared with PC-3^{AR-} cells likely contribute to these differences.
CONCLUSIONS. These studies provide new evidence that the androgen-signaling axis can be sensitized in prostate cancer cells, and have important implications for the analysis and interpretation of AR structure and function in in vitro cell systems.

KEY WORDS: **androgen ablation; androgen receptor; prostate cancer; coactivator; corepressor**

INTRODUCTION

Recent studies indicate that the androgen receptor (AR) plays an important role in many phases of prostate cancer biology, including genetic predisposition (due to the existence of polymorphic variants), disease progression, and the development of resistance to androgen-ablation therapies (AAT) [1,2]. Resistance to AAT can arise due to enhanced sensitivity of the androgen signaling axis to native and/or non-classical ligands, increased expression of the AR and/or its coregulators, or by cross-talk between growth factor/cytokine and AR signaling pathways [1,2]. Indeed, AR expression is

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retained, and may be upregulated during disease progression [3].

Analysis of AR signaling is often performed in the three most commonly available human prostate cancer cell lines, LNCaP, PC-3, and DU-145. While each of these cell lines mimic various aspects of the clinical disease (reviewed in Refs. 4–6), only LNCaP cells, derived from a moderately differentiated lymph node metastasis, have retained hormone responsiveness and express a functional AR [7–9]. LNCaP cells have been used to investigate androgen responsive genes, tumor growth in mouse xenograft models and the cross-talk between androgen and other signaling pathways [10–13]. A major limitation of the LNCaP cell line is that the AR responds equally well to androgenic and non-androgenic ligands, including estrogen, progesterone, and the AR antagonist hydroxyflutamide, as a result of a mutation in its ligand binding domain [8]. As a consequence, investigation of AR structure and function and the analysis of AR variants, identified in prostate cancer and other androgen-related diseases such as the inherited form of androgen insensitivity and Kennedy's disease [14], are often performed using transient and stable transfection of the AR negative PC-3 and DU-145 cell lines. In addition, PC-3 and DU-145 cells have commonly been used as models of prostate cancer progression, and to define the molecular mechanisms contributing to the development of androgen resistance.

The PC-3 cell line, which can be transfected with relatively high efficiency, was originally derived from a bone metastasis of human prostatic adenocarcinoma origin and is unresponsive to androgens in culture [15,16]. PC-3 cells are poorly differentiated, have general defects in mismatch repair [17], do not express p53 [18], and have a constitutively active phosphoinositide 3-kinase (PI3K)/Akt pathway [19], consistent with many of the changes observed in progression of clinical prostate cancer [17,20–25]. Although original studies found that PC-3 cells were negative for AR, ER, and PR [16,26,27], more recent studies of descendent cells obtained from the primary supplier, American Type Culture Collection (ATCC, Rockville, MD), have demonstrated significant levels of structurally normal AR mRNA, and both ERα and ERβ [28–32]. These cells have been termed PC-3^{AR+} to distinguish them from those that do not exhibit AR message (i.e., PC-3^{AR-}) [28]. The difference in AR RNA expression may reflect methylation of the AR promoter, which has previously been reported as unmethylated in PC-3^{AR+} cells but partially methylated in the PC-3^{AR-} line [33,34].

In previous studies, AR protein was not detected in PC-3^{AR+} cells by either immunoblot analysis or ligand binding assays, and endogenous AR regulated genes were not activated by treatment with the native ligand, 5α-dihydrotestosterone (DHT) [5,28,30]. In contrast, the introduction of full length AR by stable or transient transfection confers androgen responsiveness on PC-3 cells, including ligand dependent regulation of endogenous androgen responsive genes and exogenous reporter constructs [35,36]. These findings suggest that AR signaling pathways are intact in PC-3 cells despite a very low level of AR protein [37]. In the present study, we demonstrate salient differences in cell phenotype, growth rate, expression profile of known AR coregulatory proteins, and AR function between PC-3^{AR-} cells and PC-3^{AR+} cells maintained in long-term culture. Our results provide evidence that prostate cancer cells retain sensitivity to androgen signaling in the absence of detectable levels of AR protein, and have important implications for the study and interpretation of AR function in in vivo cell systems, especially in "generic" PC-3 cells.

MATERIALS AND METHODS

Cell Culture

LNCaP, DU-145, PC-3, and MDA-MB-453 human cancer cell lines were obtained from ATCC and maintained in RPMI-1640 medium (Invitrogen, San Diego, CA) supplemented with 5% fetal bovine serum (FBS) at 37°C with 5% CO_2. Cells were routinely archived on liquid nitrogen in FBS containing 10% DMSO. As described previously, PC-3 cells were obtained from ATCC on two separate occasions and designated PC-3^{AR-} or PC-3^{AR+} based on detectable levels of AR mRNA [28]. At the time of this study, cells had been passaged moderately (PC-3^{AR-}, 15–20 times) or often (PC-3^{AR-}, 40–50 times; PC-3^{AR+}, 70–80 times) from receipt.

Mycoplasma Testing

Routine mycoplasma testing was performed throughout this study as follows. DNA was extracted from 2 ml of culture medium by centrifugation and lysis in the presence of 0.4 mg/ml proteinase K, and subsequently purified using Strataclean resin™ (Stratagene, La Jolla, CA) according to the manufacturers protocol. PCR was performed using mycoplasma specific sense (5′-ACTCCTACGGGAGGC-AGCAGTA-3′) and antisense (5′-TGCACCATCTGT-CACTCTGTTAACCTC-3′) oligonucleotide primers. PCR products were resolved on a 2% agarose gel and analyzed for the specific 715-bp mycoplasma band. Cultured cells were free from mycoplasma infection throughout this study.

Cell Proliferation Assays

PC-3 cells were seeded in 96-well plates at 2,500 cells/well and cultured in RPMI-1640 medium

with 5% FBS for 12 hr. Cells were washed with PBS and the medium replaced with RPMI-1640 medium containing FBS (0.25–5%) or dextran coated charcoal (DCC) treated serum (5%) with or without the addition of specific ligands. MTT assays [38] were initiated at 24 hr intervals by the addition of 10 µl of aqueous MTT (5 mg/ml) to each well and incubation for 4 hr at 37°C. A solution of 20% SDS and 0.02 M HCl (100 µl) was added to each well and the plate rested overnight in complete darkness at room temperature. Plates were analyzed using a BioRad 450 Microplate Reader set to dual wavelength (570/655 nm). Standard curves for each subline were generated by assaying 96-well plates seeded with 0–50,000 cells/well in RPMI-1640 containing 5% FBS and incubated for 12 hr.

Genotyping

Genetic profiling was performed using a discriminating system for human identification applications (AmpFLSTR Profiler Plus™ PCR Amplification Kit, Applied Biosystems, Inc.), which amplifies nine tetranucleotide short tandem repeat (STR) loci (D3S1358, D5S818, D8S1179, D13S317, D18S51, D21S11, FGA, and vWA) and the Amelogenin locus (which discriminates different product lengths from the X and Y chromosome). DNA was extracted from cells growing at log-phase and PCR reactions performed in accordance with the manufacturers instructions. Each amplification product of 0.8 µl was diluted with 10 µl formamide containing a fluorescent ROX-labeled size standard (GS-500; Applied Biosystems, Inc.) at a 20:1 ratio. The diluted products were run on an ABI 3700 Capillary Electrophoresis DNA Analyzer (Applied Biosystems, Inc.) and analyzed with GeneScan v3.5 software (Applied Biosystems, Inc.).

Karyotyping

Metaphase chromosomes were prepared from cultured cells according to standard protocols, with a few modifications. Briefly, cells were blocked in metaphase with Colcemid (0.05 ng/ml) for 1–2 hr before hypotonic swelling in a 4:1 mixture of 0.075 M KCl and 1% sodium citrate. Fixation was performed using a 3:1 mixture of methanol and glacial acetic acid on ice-cold water-rinsed slides. Chromosomes were stained with Leishman's stain. Metaphase chromosomes were digitally imaged and karyotyped with a CytoVysion System (Applied Imaging, Santa Clara, CA). The karyotype was assayed for five metaphase spreads of each cell type. Abnormalities were listed as clonal when present in at least two cells. The composite karyotype of each cell line was described using the ISCN conventions [39].

AR Transactivation Assay

PC-3 cells were seeded in 96-well plates in RPMI-1640 medium containing 5% FBS at a density of 15,000 cells/well and incubated for 16 hr. For assays of AR transactivation, a plasmid mix included 0–20 ng of wtAR expression vector, pcDNA3.1-wtAR$(CAG)_{21}$ [40], and 100 ng of the androgen responsive probasin reporter construct, ARR3-tk-luciferase [41] was prepared. For cofactor studies, the plasmid mix contained 0.5 ng of pcDNA3.1-wtAR$(CAG)_{21}$ expression vector, 100 ng of the ARR3-tk-luc, and 50 ng of coregulator expression vector (pCMX:hfSMRT, pSG5:GRIP1, pSG5:mZac1, pcDNA-BRCA1). In cells that did not receive cofactor, an equivalent molar amount of empty expression vector was used to balance promoter usage, whereas total transfected DNA was balanced using the promoterless pCAT-basic or prokaryotic pBS(sk−) plasmids. Analysis of AR variant F671I was performed using pCMV-wtAR and pCMV-F671I expression vectors described previously [42]. For transfection, cells were washed with serum and phenol-red-free RMPI-1640 and transfected for 4 hr with the appropriate vectors mixed with 0.4 µl LipofectAMINE 2000™ (Gibco-BRL, Melbourne, Vic., Australia) per well according to the manufacturer's protocol. Following transfection, the reaction mix was carefully removed, and cells overlaid with RPMI-1640 medium containing 5% DCC treated FBS supplemented with 0.001–100 nM of the appropriate ligand. Cells were incubated for 36 hr and then harvested directly from plates with 50 µl passive lysis buffer (Promega Corporation, Madison, NY) per well. Luciferase activity was determined in 25 µl samples from each well using the Luciferase™ Reporter Gene Assay Kit (Promega Corporation) and a plate reading luminometer (Top Count™, Packard, Mount Waverley, ACT, Australia).

RNA Preparation

For each cell line, RNA was prepared from three independent cultures of 1×10^6 cells grown in 6-well culture dishes in RPMI-1640 supplemented with 5% FBS using the RNeasy Mini Kit (Qiagen) according to the manufacturers instructions. The integrity of each RNA sample was assured by gel electrophoresis on a 1% agarose-MOPS gel containing 18% formaldehyde. RNA was treated with DNase, extracted using phenol:chloroform:isoamyalcohol, precipitated with ethanol/sodium acetate and reverse transcribed using SuperscriptII™ reverse transcriptase (Gibco-BRL) according to manufacturers instructions. Duplicate aliquots of each RNA preparation were treated without the addition of the reverse transcriptase. The integrity of each cDNA preparation and the absence of contaminating genomic DNA were determined by PCR

amplification of β-actin using oligonucleotide primers (Table I) that can amplify both the β-actin cDNA (202 bp product) and genomic DNA (314 bp product).

Quantitative Real-Time PCR (QR-PCR)

To determine relative RNA levels, QR-PCR was performed using 10 μl of 2× SYBR Green PCR Mastermix (Applied Biosystems, Inc.), 8 μl of a 1/100 dilution of cDNA and 5 pmol of specific sense and antisense primers (Table I) with a Rotor-Gene 2000 (Corbett Research, Mortlake, NSW, Australia) instrument using the following PCR parameters: 94°C for 10 min; 50 cycles of 94°C for 15 sec, 60°C for 15 sec, and 72°C for 30 sec. Instrument melt curve analysis and gel electrophoresis were used to assure amplification of a single product. The relative expression level was determined by normalization of cycle threshold values for each gene to those of β-actin in each sample using Rotor-Gene software. The average value derived from triplicate RNA samples is presented.

Immunoblot Analysis

To analyze endogenous AR level, MDA-MB-453, LNCaP, PC-3^{AR+}, and PC-3^{AR-} cells were seeded in 6-well plates (35 mm in diameter) in serum- and phenol-red-free RPMI-1640 medium containing 5% DCC treated FBS at a density of 0.3×10^6 cells/well. After a 72-hr incubation, the medium was removed and overlaid with serum- and phenol-red-free RPMI-1640 medium containing 5% DCC treated FBS supplemented with 1 nM DHT or ethanol vehicle. Cells were incubated for a further 36 hr and harvested as detailed below. To analyze AR levels following transfection of an AR expression vector, cells were seeded in 6-well dishes (35 mm) at a concentration of $0.5–1.0 \times 10^6$ cells/well and grown for 24 hr in RPMI-1640 medium containing 5% FBS. Cells were transfected as detailed for transactivation assays, using an equivalent amount of AR plasmid DNA/cell [i.e., 0.1–20 ng pcDNA3.1-wtAR$(CAG)_{21}$/15,000 cells], and incubated for 36 hr in serum- and phenol-red-free RPMI-1640 medium containing 5% DCC treated FBS supplemented with the appropriate concentration of DHT or ethanol vehicle. Cells were lysed using a standard RIPA buffer supplemented with 2 mM PMSF, 0.01 mM Aprotinin (Sigma, St. Louis, MO), 100 mM NaF, 10 mM $Na_4P_2O_7$, and 2 mM Na_3VO_4. Protein concentration was determined in each sample using the Bradford protein assay kit (BioRad, Hercules, CA). Twenty micrograms of total cellular protein from each sample was electrophoresed on a 6% SDS–PAGE, transferred to Hybond-C or Hybond-P membrane (Amersham Pharmacia Biotech, Piscataway, NJ) and immunostained using specific AR antisera (U407) and with actin I-19 antisera (Santa Cruz Biotechnology, Inc.). Immunoreactivity was detected using the appropriate HRP-conjugated anti-rabbit IgG or HRP-conjugated anti-goat IgG antibodies (Santa Cruz Biotechnology, Inc.) using either ECL Western blotting reagents (Amersham Pharmacia Biotech) or Chemiluminescence Luminol Reagent (Santa Cruz Biotechnology, Inc.) according to the manufacturer's protocols. Immunoreactivity was quantified using a Fluor-S™ Multi-Imager MAX instrument (Bio-Rad, Hercules, CA).

RESULTS

Morphology and Growth Characteristics

Phase contrast microscopy demonstrated that the PC-3^{AR-} cell line has an elongated epithelial morphology characteristic of the parental PC-3 cell line in culture [15]. In contrast, late passage, PC-3^{AR+} cells have an altered morphology with rounded cells adopting a cobble-stone appearance, possibly reflecting a dedifferentiated phenotype (Fig. 1A). PC-3^{AR-} cells did not exhibit any changes in morphology when passaged an additional 40 times (Fig. 1A). The different morphology and growth characteristics of PC-3^{AR-} and PC-3^{AR+} cells (see below) were not attributable to mycoplasma infection (Fig. 1B), which has previously been shown to alter the expression of genes in

TABLE I. Oligonucleotide Primers for Quantitative Real-Time PCR (QR-PCR)

Message	Sense oligonucleotide (5′–3′)	Antisense oligonucleotide (5′–3′)
AR	AGCCATTGAGCCAGGTGTAGTGTG	GTGAAGGATCGCCAGCCCAT
β-Actin	GCCAACACAGTGCTGTCTGG	TACTCCTGCTTGCTGATCCA
BRCA1	AGAAACCACCAAGGTCCAAAGC	TGCCAAGGGTGAATGATGAAAG
GRIP1	CACAGCAGTTTCCATTTCCTCCAA	CCCTGCGCCCATCCATTTAT
Hic5	CATTCTCCTCTTCCAGCGGT	AGGCCATCAGTCTATCCAGC
SMRT	TTGGGTGGTGGTGAGGACGGTA	TTGCTGAAGAAGGCTGGCGG
Zac1	GTCCATAGCCTCACCCTCAG	CAGCATTTCCCTTAGCCAGA

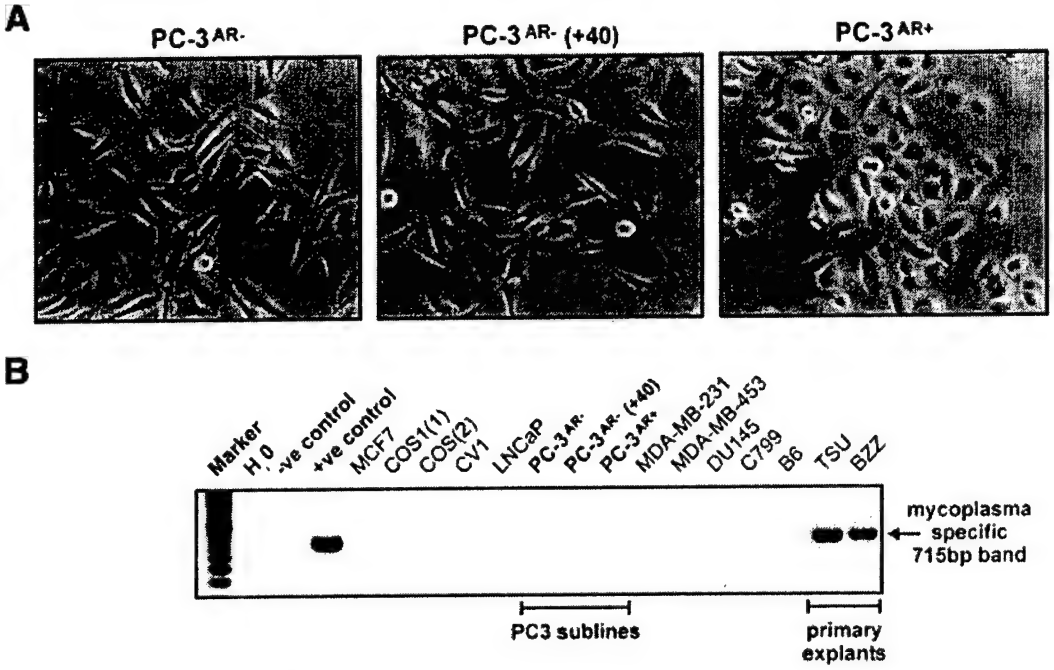

Fig. 1. Morphology of PC-3 sublines and mycoplasma testing of cultured cells. **A**: Morphology: cells growing in RPMI-1640 medium supplemented with 5% FCS were imaged 2 days after passage at 1/5 (PC-3^{AR-}) or 1/10 (PC-3^{AR+}) in flasks (75 cm^2) using a phase contrast microscope and a digital camera. Distinct differences in morphology of PC-3^{AR-} and PC-3^{AR+} cells are observed. **B**: Mycoplasma testing: DNA was extracted from culture medium of confluent cells and amplifed by PCR reaction using degenerate mycoplasma specific primers for the 715 bp specific mycoplasma band. All cells were mycoplasma free throughout this study.

prostate cells [43], and the growth rate, morphology and behavior of cultured cell lines (reviewed in Refs. [44–46]).

PC-3^{AR+} cells exhibited an increased rate of proliferation compared to PC-3^{AR-} cells (Fig. 2A), were more capable of sustained proliferation in standard and low serum conditions (Fig. 2B), and were more robust in culture, exhibiting greater adherence to culture flasks, and resistance to detachment and lysis (data not shown). Proliferation of PC-3^{AR-} and PC-3^{AR+} cells was not altered by the addition of DHT or the AR antagonist hydroxyflutamide (Fig. 2A).

Genotype and Karyotype

Microsatellite genotyping of PC-3^{AR-} and PC-3^{AR+} cells did not detect allele size variation at any of the discriminating loci examined, indicating a common clonal origin of these sublines (Table II). In contrast, LNCaP cells exhibited distinct allele lengths at 8/9 STR loci (Table II). The lack of a 113 bp fragment for the Amelogenin Y chromosome locus in both PC-3 sublines is consistent with the absence of the Y chromosome observed by karyotype analysis in this (see below) and previous studies [15].

Karyotypic analysis of PC-3^{AR-} and PC-3^{AR+} cells determined that the median (range) chromosome number was 62 (62–63) and 58 (57–62), respectively (Table III). This is consistent with a previous report of a reduction in modal chromosome number from 62 to 58 in PC-3 cells with increasing passage (from 5 to 30) [15]. Autosomes 2, 3, 4, 5, 12, and 15, and the Y chromosome were characteristically absent from both PC-3^{AR-} and PC-3^{AR+} sublines (Table III), as reported for the original isolate of PC-3 cells [47]. PC-3^{AR+} cells exhibit a greater number of marker chromosomes than the PC-3^{AR-} line (i.e., 13 compared with 8 markers).

PC-3^{AR+} Cells Express AR RNA and Detectable AR Protein

In a previous study, we utilized RNAse protection assays to demonstrate that PC-3^{AR+} but not PC-3^{AR-} cells express AR RNA [28]. We confirmed this result here using RT-PCR analysis (Fig. 3A). However, immunoblot analysis using AR antisera (U407 [48]) did not demonstrated any significant level of AR protein in either PC-3^{AR-} or PC-3^{AR+} cells compared with LNCaP and MDA-MB-453 cell lines (Fig. 3B). We, therefore, utilized QR-PCR analysis to determine the

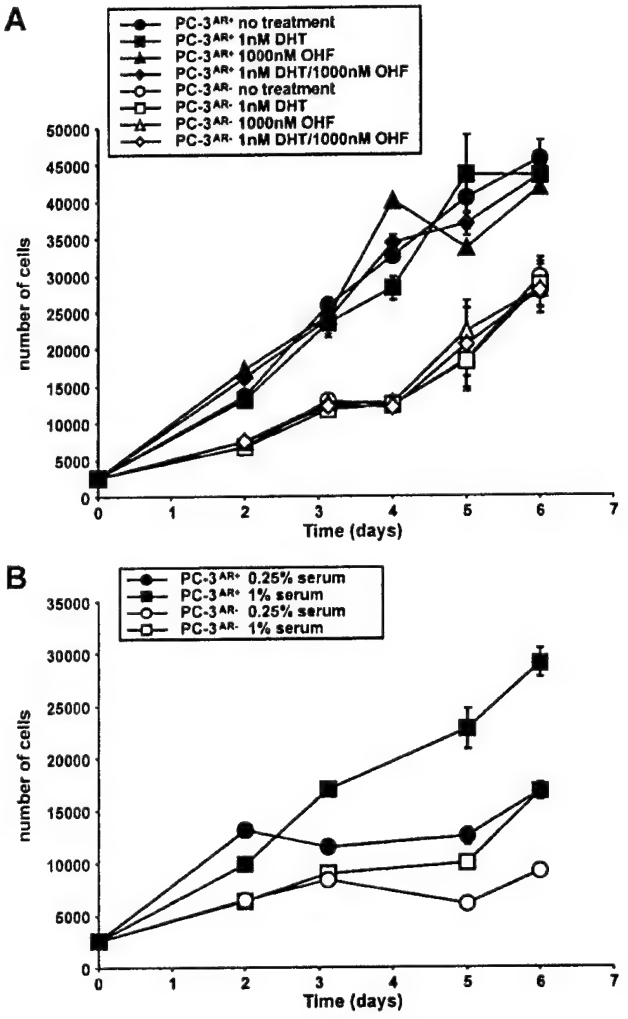

Fig. 2. Analysis of PC-3 subline proliferation using MTT assays. Standard curves (not shown) were generated 12 hr after seeding at 0–50,000 cells/well using MTT assays and linear regression analysis. For cell growth analysis, MTT assays were performed at the indicated time points and the number of cells/well determined from standard curves. PC-3^{AR+} cells approached, but did not reach, logarithmic growth, presumably due to limitations in culture of these very rapidly growing cells in 96-well dishes. **A**: While PC-3^{AR+} cells proliferate more rapidly than PC-3^{AR-} cells, dihydrotestosterone (DHT), and hydroxyflutamide had no effect on growth of either cell line. **B**: PC-3^{AR+} cells grow better than PC-3^{AR-} cells in low serum conditions.

relative level of AR expression in PC-3^{AR+} cells compared to other androgen responsive and non-responsive cell lines. AR RNA expression in PC-3^{AR+} cells was comparable to that of the MDA-MB-453 breast cancer cell line, which is androgen responsive in culture and contains AR protein detectable by immunoblot analysis, but significantly lower than observed in LNCaP cells (Fig. 4A,B). AR RNA was not detected in AR negative DU-145 cells (Fig. 4B). PC-3^{AR-} cells passaged an additional 40 times did not exhibit AR RNA or protein by the above analyses (data not shown).

Endogenous AR in PC-3^{AR+} Cells Does Not Activate an Androgen Responsive Reporter

To determine whether AR expression in PC-3^{AR+} cells results in sufficient AR protein to activate androgen responsive genes, PC-3^{AR-}, PC-3^{AR+}, LNCaP, and MDA-MB-453 cells were transiently transfected with the androgen responsive probasin-luciferase promoter and treated with DHT. In contrast to LNCaP (Fig. 5A) and MDA-MB-453 cells (Fig. 5B), DHT treatment did not result in an increase in luciferase signal in either PC-3^{AR-} or PC-3^{AR+} cells containing the reporter alone (Fig. 5C). Moreover, neither AR coactivators, GRIP1 and mZac1 [49,50], nor the corepressor SMRT [51,52], affected luciferase activity in either PC-3^{AR-} or PC-3^{AR+} cells in the presence of DHT (Fig. 5C). These data indicate that any endogenous AR expressed in either PC-3^{AR-} or PC-3^{AR+} cells is inactive and/or does not reach an expression level sufficient for activation of androgen responsive genes. This is consistent with the inability of AR agonists or antagonists to modulate proliferation of PC-3^{AR+} cells (Fig. 2). In contrast, DHT was able to elicit a marked increase in reporter gene activity in both PC-3^{AR-} and PC-3^{AR+} cells cotransfected with a wild-type AR expression vector (Fig. 5C). While the activity in vehicle treated PC-3^{AR+} cells cotransfected with AR was higher than in cotransfected PC-3^{AR-} cells, DHT elicited a much greater relative increase in luciferase activity over basal signal in PC-3^{AR+} cells [99 (±5) fold] compared to PC-3^{AR-} cells [16 (±2)-fold] (Fig. 5C).

PC-3^{AR+} Cells Exhibit Greater Ligand Dependent Activation and Enhanced Response to AR Coregulators

The transactivation response of transfected AR in PC-3^{AR+} was increased compared to PC-3^{AR-} cells at all concentrations of DHT examined (0.001–100 nM; Fig. 6A). Nonetheless, when plotted as the percent activity induced by 1 nM DHT in each cell line, the dose–response curves were identical (Fig. 6B), demonstrating that the lower activity in PC-3^{AR-} cell line is not due to an altered capacity of transfected AR to bind agonists in these cells. A similar level of AR protein was clearly discernable by immunoblot analysis in transfected PC-3^{AR-} and PC-3^{AR+} cells in the absence of DHT (Fig. 6C). In contrast, DHT may result in increased AR protein in PC-3^{AR+} cells (Fig. 6C), thereby contributing in part to the increased transactivation activity of exogenous AR in this line compared with PC-3^{AR-} cells. Transfection of increasing amounts of AR expression vector resulted in a linear increase in

TABLE II. Genotype Analysis of PC-3^{AR-}, PC-3^{AR+}, and LNCaP Cells

Locus	Location	Motif	Allele length (bp)			
			Normal range	LNCaP	PC-3^{AR-}	PC-3^{AR+}
D3S1358	3p	TCTA(TCTG)$_{1-3}$(TCTA)	114–142	130/130	130/130	130/130
FGA	4q28	(TTTC)$_3$TTTTTTCT(CTTT)$_n$-CTCC(TTCC)$_2$	219–267	223/227	243/342	243/342
D5S818	5q21-31	(AGAT)$_n$	135–171	147/151/155	159/159	159/159
D7S820	7q11.21-22	(GATA)$_n$	258–294	270/278	266/278	266/278
D8S1179	8	(TCTR)$_n$	128–168	144/152	148/148	148/148
vWA	12p12-pter	TCTA(TCTG)$_{3-4}$(TCTA)$_n$	157–197	177/185	181/181	181/181
D13S317	13q22-31	(GATA)$_n$	206–234	218/226	222/222	222/222
D18S51	18q21.3	(AGAA)$_n$	273–341	281/285	293/297	293/297
D21S11	21	(TCTA)$_n$(TCTG)$_n$[(TCTA)$_3$TA-(TCTA)$_3$TCA(TCTA)$_2$ TCCATA](TCTA)$_n$	189–243	203/207/221	207/217	207/217
Amelogenin	X:p22.1-22.3		107	107	107	107
Amelogenin	Y:p11.2		113	113	n.p.[a]	n.p.[a]

DNA was extracted from each cell line and genetic profiling performed using a discriminating system for human identification applications, which amplifies nine tetranucleotide short tandem repeat (STR) loci and the amelogenin locus (which discriminates different product lengths from the X and Y chromosome). Allele lengths were determined against a fluorescent ROX-labeled size standard by capillary electrophoresis and GeneScan software analysis.
[a]Consistent with the absence of a Y chromosome in PC-3 cells as previously reported [5,15].

TABLE III. Composite Karyotype of PC-3^{AR-} and PC-3^{AR+} Cells*

PC-3^{AR-}	PC-3^{AR+}
62–63,XX,–Y	57–62,XX,–Y
–1	add(q10),add(p21)
t(2;3)p(2?3;q1?3)	–2,t(2;3)p(2?3;q1?3)
add(3)(q1?3)	–3,add(3)(q1?3)
del(4)(q21q31) × 2,add?(4)(q10)	–4,del(4)(q21q31) × 2
–5,–5,add(5)(q11.2)	–5,–5,add(5)(q11.2)
add(6)(q27)	add(6)(q27) × 2
–8,–8	–8,del(8)(p21p23)
–9	–9
–10,add(10)(p11.2) × 2	–10,add(10)(p11.2) × 2
+11,add(11)(p15),der(11)t(5:11)(q13;p15)	+11,add(11)(p15),der(11)t(5:11)(q13;p15)
add(12)(q24),add(12)(p1?3),der(12)add(12)q24),-del(12)(p?12p?13)	–12,add(12)(q24),add(12)(p1?3)
–13	–13,–13
	–14
–15,der(15)t(5;15)(q13;p?13),i(15)(q10)	–15,add(15)(p11.2),der(15)t(5;15)(q13;p?13)
–16	–16,–16
–17,–17	–17
del(18)(q12q22)	del(18)(q12q22) × 2
add(19)(p13.3)	–19,add(19)(p13.3) × 2
+20	
+21	add(21)(p11.2)
–22	–22
+m1 × 2,+m2,+m3,+m4,+m6,+m18	+m1,+m3,+m5 × 2,+m6 × 2,+m11,+m12,+m13,+m14,+m16,+m17

Metaphase chromosomes were prepared from cultured cells and digitally imaged. The karyotype was assayed for five metaphase spreads of each cell type using the CytoVysion system (Applied Imaging, Santa Clara, CA). Abnormalities were listed as clonal when present in at least two cells.
*The composite karyotype of each cell line is described using the ISCN conventions [39].

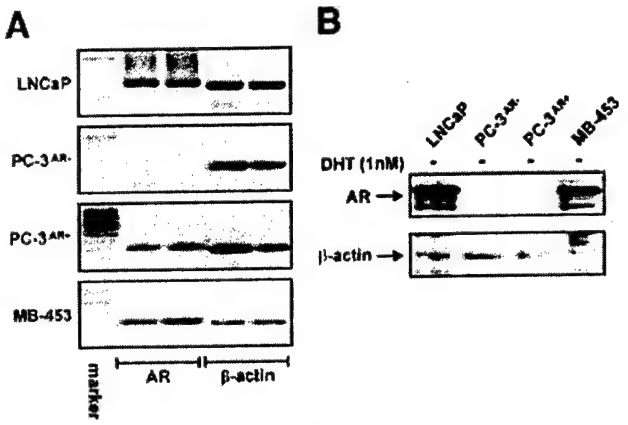

Fig. 3. Expression of AR message and protein in human cancer cell lines. **A**: RT-PCR analysis demonstrated AR expression in PC-3^{AR+}, LNCaP, and MDA-MB-453 cells, but not in the PC-3^{AR-} line. **B**: Immunoblot analysis for β-actin (I-19 antisera, Santa Cruz Biotechnology, Inc.) and AR (U407 [48]) antisera demonstrated little AR protein in either PC-3^{AR-} or PC-3^{AR+} cells compared with LNCaP and MDA-MB-453 lines.

detectable AR protein in both PC-3^{AR-} and PC-3^{AR+} cells (Fig. 6D,E), but only PC-3^{AR+} cells exhibited a similar relationship between the amount of transfected AR plasmid and reporter gene activity (Fig. 6F). These results suggest that key mediators of AR function, such as coregulators or members of the basal transcription machinery [53], could be limiting in PC-3^{AR-} cells. To test this hypothesis, PC-3^{AR-} and PC-3^{AR+} cells were cotransfected with wtAR and known AR coactivators, GRIP1, mZac1, and BRCA1 [49,50,54]. While each of the AR coregulators was able to increase AR transactivation in both PC-3^{AR-} and PC-3^{AR+} cells, they elicited a greater net increase in AR activity in PC-3^{AR+} cells (34,000–507,000 counts) compared with PC-3^{AR-} cells (3,900–15,500 counts) (Fig. 6G). The maximum activity in the PC-3^{AR-} line in the presence of these coregulators did not approach that observed for AR in the absence of coregulators in PC-3^{AR+} cells. However, the fold increase in signal over wtAR cotransfected with vehicle was greater for each coactivator in PC-3^{AR-} compared to PC-3^{AR+} cells (Fig. 6G).

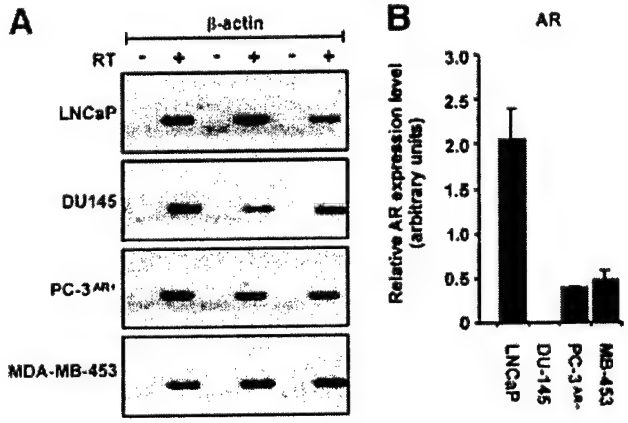

Fig. 4. Quantitative analysis of AR RNA in human cancer cell lines. **A**: Integrity of cDNA preparations determined by PCR for β-actin from triplicate RNA samples treated with (+) or without (−) reverse transcriptase. **B**: Quantitative real-time PCR (QR-PCR) analysis demonstrating that PC-3^{AR+} cells express equivalent amounts of AR RNA to the androgen responsive MDA-MB-453 cell line, but less than LNCaP cells. Data is presented as the relative expression level in arbitrary units and represents the mean (±SEM) of the specific signal compared to β-actin from the three independently isolated RNA preparations shown in (A).

AR Coregulators Have Increased Expression in PC-3^{AR+} Cells

The above results suggest that the level of specific AR coregulators may be increased in PC-3^{AR+} compared to the PC-3^{AR-} cells. Indeed, an approximately threefold greater expression of GRIP1 and a twofold greater expression of Zac1 and SMRT in PC-3^{AR+} cells was observed using QR-PCR (Fig. 7). Levels of the AR coregulator, Hic5/ARA55 [55] were similar in both cell lines, while BRCA1 expression was very low to undetectable (Fig. 7). There was no change in the level of these AR coregulators in PC-3^{AR-} cells following an additional 40 passages (Fig. 7). An increase in the level of AR coregulators in PC-3^{AR+} cells is consistent with the reduced fold coactivation of AR to these cofactors compared to PC-3^{AR-} cells (see Fig. 6G above). Moreover, as higher levels of p160 coactivators result in increased sensitivity of androgen signaling in vitro and in vivo [56], an increase in GRIP1 levels may explain the higher basal activity from the androgen responsive reporter gene in transfeced PC-3^{AR+} cells in the absence of ligand (see Fig. 5C above). However, as both PC-3 sublines and LNCaP cells expressed significant levels of most factors tested (Fig. 7), other undetermined AR coregulators are likely to be responsible for the increased AR transactivation capacity in PC-3^{AR+} cells.

The Phenotype of AR Variants Is Masked in PC-3^{AR-} Cells

AR variants identified in human prostate cancer generally exhibit a gain-of-function phenotype [1,3,57–59]. The most commonly characterized AR variants have structural changes in the ligand-binding pocket that allows promiscuous binding and activation by non classical ligands [60–62]. In contrast, a less well characterized subset such as AR-F671I, which has a disrupted putative protein–protein interaction domain on the surface of the receptor [63], appear to depend on the level of unknown or poorly characterized cellular cofactors for increased transactivation function

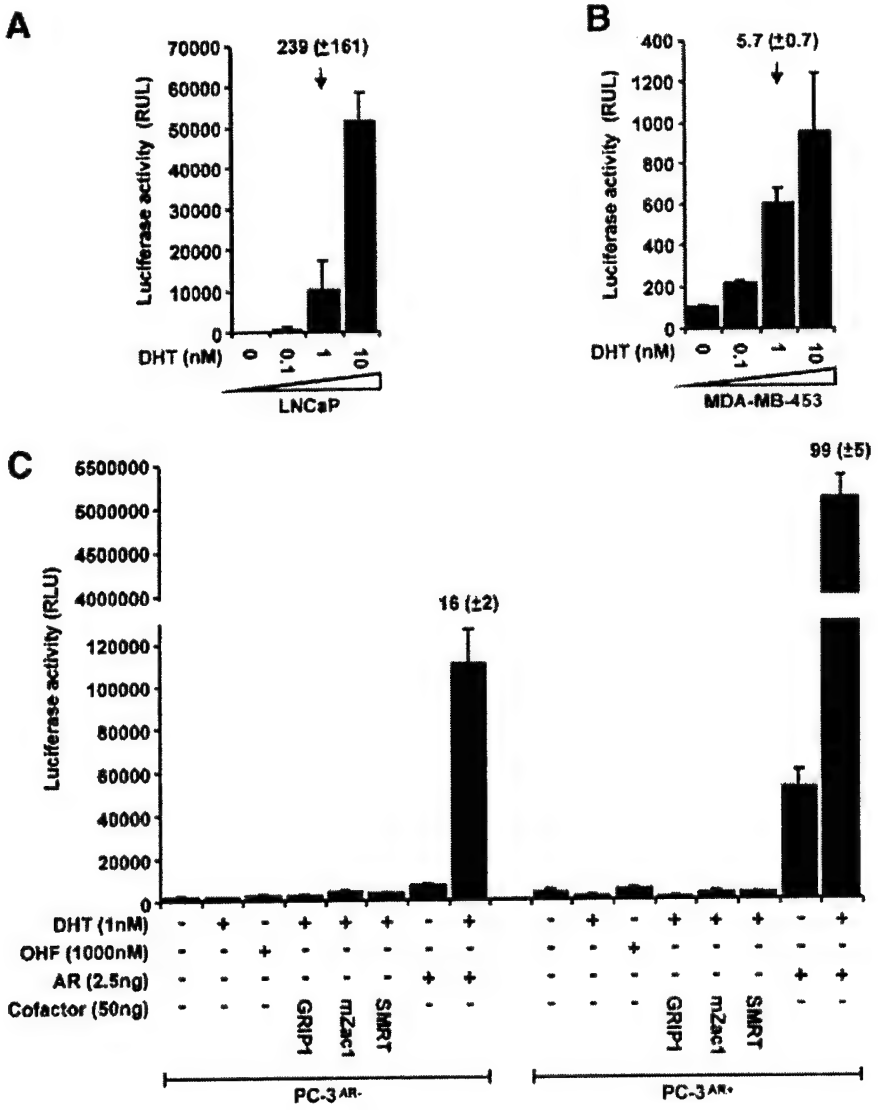

Fig. 5. Activation of an androgen responsive reporter by endogenous AR in human cancer cell lines. **A**: LNCaP and (**B**) MDA-MB-453 cells were transfected with the ARR3-tk-luciferase (probasin) reporter gene and incubated in the absence or presence of DHT (0.1–10 nM) as indicated. Data is presented as luciferase counts in relative light units and represents the mean (±SEM) of six individually transfected wells. The average fold (±SEM) increase in AR activity of 1 nM DHTover vehicle alone is indicated in each case. **C**: PC-3^{AR-} and PC-3^{AR+} cell lines were transfected with the ARR3-tk-luciferase reporter with or without AR and/or coregulator expression vectors, and incubated with or without DHT (1 nM) or hydroxyflutamide (OHF; 1,000 nM) as indicated. Data is presented as luciferase counts in relative light units and results represent the mean (±SEM) of five individually transfected wells. While activity in untransfected cells was equivalent in both cell lines, the average fold (±SEM) increase in activity of transfected AR by 1 nM DHTover vehicle alone was greater in PC-3^{AR+} than PC-3^{AR-} cells.

[42,53,63,64]. In this study, a gain-of-function for the AR-F671I prostate cancer variant was significantly more obvious in PC-3^{AR+} than in PC-3^{AR-} cells (Fig. 8). This data supports the above conclusion that PC-3^{AR+} cells have an altered cellular milieu of AR coregulators.

DISCUSSION

In this study, we report salient differences in cell morphology, expression of endogenous AR, proliferation rate, and function of transfected AR between the two PC-3 sublines, PC-3^{AR-} and PC-3^{AR+}. The PC-3 cell line was originally described as consisting of "epithelial-like cells and spherical grapelike aggregates" [15]. In contrast, we observed exclusively epithelial-like cells in the PC-3^{AR-} line and rounded cells that adopt a cobble-stone appearance in the PC-3^{AR+} line. Genotype and karyotype analysis confirmed the clonal origin of PC-3^{AR-} and PC-3^{AR+} cells, suggesting that the two sublines diverged from a common progenitor

or were independently selected during culture. Previous studies have demonstrated that PC-3 and other prostate cancer cells display heterogeneity during culture, exhibiting different phenotypes that may reflect selection pressure(s) during long-term culture [65,66].

We previously reported that the AR in PC-3^{AR+} cells could be detected by immunohistochemistry using

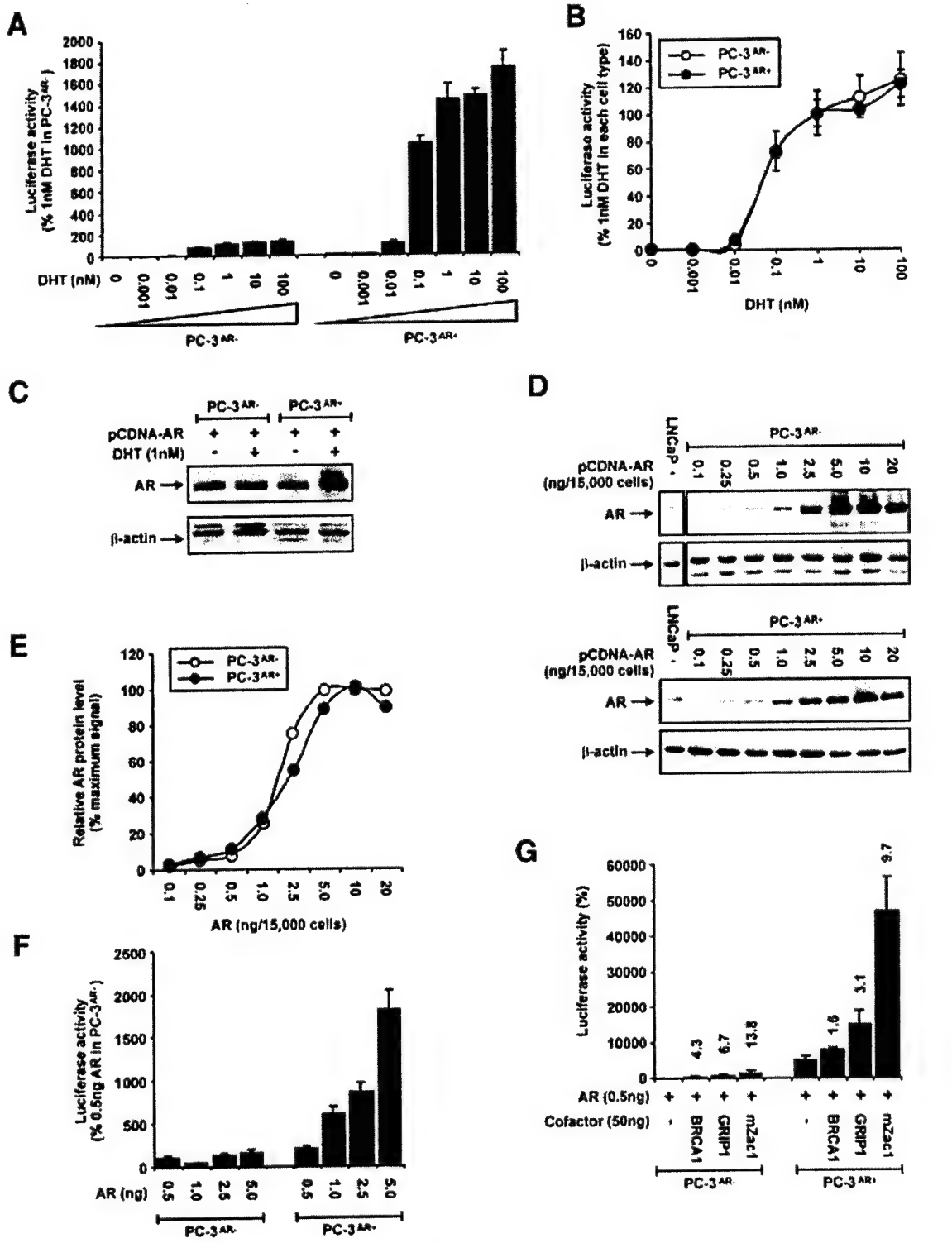

Fig. 6.

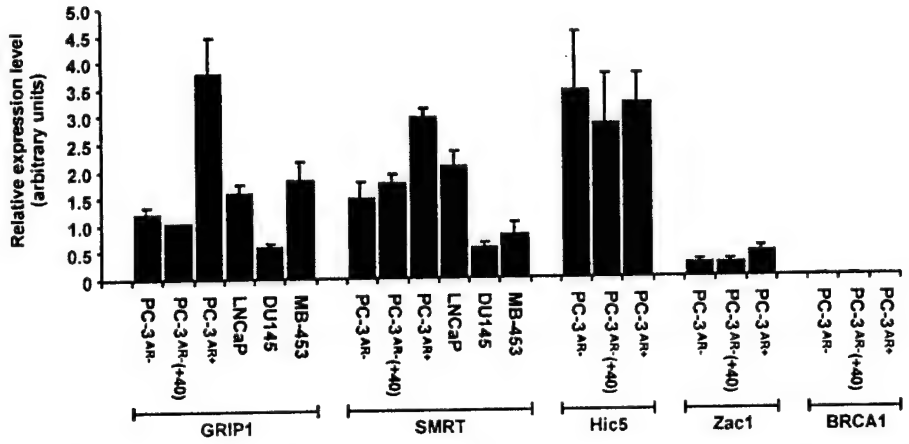

Fig. 7. QR-PCR analysis of AR cofactor expression in human cancer cell lines. QR-PCR analysis of GRIP1, SMRT, Hic5, Zac1, and BRCA1 expression in prostate (PC-3^{AR-}, PC-3^{AR-}(+40), PC-3^{AR+}, LNCaP, and DU-145) and breast (MDA-MB-453) cancer cell lines demonstrated increasing levels of some coregulators in PC-3^{AR+} compared with PC-3^{AR-} cells. Data is presented as the relative expression level in arbitrary units and represents the mean (±SEM) of the specific signal compared to β-actin for three independently isolated RNA preparations.

antisera specific for two distinct AR epitopes (U407, R489) [48], but not by radio-ligand binding or immunoblot analysis [28]. This observation suggested that the AR message is translated in PC-3^{AR+} cells but the receptor protein does not accumulate at sufficient levels, or is degraded following cell fractionation, thereby preventing detection by immunoblotting or radio-ligand binding. The absence of radio-ligand binding in PC-3^{AR+} cells could not be explained by structural alterations in the *AR* gene [16,29]. In the current study, AR protein was similarly not detected in PC-3^{AR-} cells or in PC-3^{AR+} cells by Western blot following long-term culture. In addition, neither DHT nor receptor antagonists affect the growth of either cell line. In LNCaP cells, inhibition of proteasome function markedly enhances AR protein levels [67]. It is conceivable, therefore, that altered AR degradation or turnover in PC-3^{AR+} cells could be responsible for the low AR protein/RNA ratio. Moreover, if a low steadstate level of AR protein is present in PC-3^{AR+} cells it does not reach a level sufficient to activate transcription of an androgen responsive reporter gene, potentially due to the relatively high levels of endogenous AR coregulators such as SMRT.

The transactivation activity of exogenous AR is consistently greater in PC-3^{AR+} than in PC-3^{AR-} cells, without altered sensitivity to activation by DHT. Moreover, the increased transactivation capacity of gain-of-function AR variants such as those that affect cofactor recruitment, was apparent in PC-3^{AR+} but not PC-3^{AR-} cells, possibly because only the PC-3^{AR+} line exhibits a linear increase in reporter gene activity with increasing amounts of transfected AR. These data suggest that the increased transactivation capacity of PC-3^{AR+} cells is an important factor in assessing AR function. In particular, the functional consequences of

Fig. 6. AR transactivation activity for increasing ligand and transfected AR plasmid in PC-3^{AR-} and PC-3^{AR+} cell lines. **A**: AR transactivation capacity was greater for all concentrations of DHT (0.001–100 nM) in PC-3^{AR+} compared PC-3^{AR-} cells transfected with 0.5 ng of AR expression vector and the ARR3-tk-luciferase reporter construct. Data is presented as the percentage luciferase activity induced by 1 nM DHT in PC-3^{AR-} cells and represent the mean (±SEM) of five individually transfected wells. **B**: Data from (A) plotted for each cell type as percentage activity observed in the presence of 1 nM DHT demonstrates that the sensitivity of transfected AR to ligand is similar in PC-3^{AR-} and PC-3^{AR+} cells. **C**: AR protein was observed by immunoblot analysis with AR (N-20) antisera (Santa Cruz Biotechnology, Inc.) in untransfected PC-3^{AR+} but not PC-3^{AR-} cells. Similar levels of AR protein were observed in PC-3^{AR-} and PC-3^{AR+} cells transfected with AR expression vector. **D**: Immunoblot analysis of AR and β-actin in lysates of PC-3^{AR-} and PC-3^{AR+} cells transfected with 0.1–20 ng of AR expression vector per 15,000 cells (as indicated) using actin (I-19) and AR (N-20) antisera (Santa Cruz Biotechnology, Inc.). **E**: AR expression relative to β-actin determined from immunoblots shown in (D) determined with an imaging densitometer demonstrates that AR is successfully translated in both cell lines **F**: AR transactivation capacity induced by increasing amounts of transfected AR expression vector (0.5–5 ng) in the presence of 1 nM DHT was linear only in PC-3^{AR+} cells. Data is presented as the percent luciferase activity relative to 0.5 ng of transfected AR in PC-3^{AR-} cells and represent the mean (±SEM) of five individually transfected wells. **G**: AR coactivation by BRCA1, GRIP1, and mZac1 in PC-3^{AR-} and PC-3^{AR+} cell lines. Cells were transfected with 0.5 ng of the AR expression vector, and the ARR3-tk-luciferase reporter, with or without 50 ng of cofactor expression vectors as indicated. Data is presented as the percentage luciferase activity induced by 1 nM DHT in PC-3^{AR-} cells and represent the mean (±SEM) of five individually transfected wells. For each cofactor, the mean fold increase in activity relative to AR + DHT alone was greater in PC-3^{AR-} than in PC-3^{AR+} cells, although the absolute increase in reporter gene activity was much greater in PC-3^{AR+} cells.

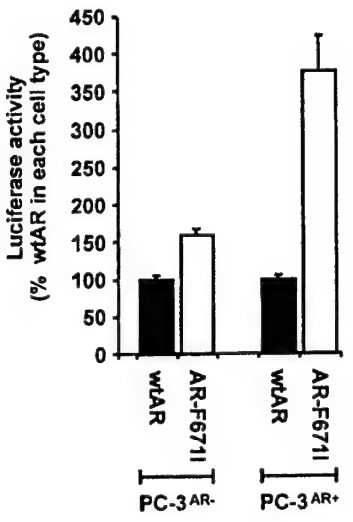

Fig. 8. Transactivation activity of AR variants in PC-3^{AR-} and PC-3^{AR+} cells. **A, B**: Transactivation capacity of AR containing different polyQ repeat length alleles (n = 9, 16, 21, 26, 29, and 42) as indicated in PC-3^{AR-} (A), and PC-3^{AR+} (B) cells transfected with 2.5 ng of each AR expression vector and the MMTV-luciferase reporter construct in the presence of 1 nM DHT. Data is presented as percent luciferase activity for wtAR carrying 21 CAG repeats in each cell type and represents the mean (±SEM) of six individually transfected wells. A relationship between polyQ repeat length and AR transactivation function was only observed in PC-3^{AR+} cells. **C**: Increased transactivation capacity of the F671I AR variant compared to wtAR was more apparent in PC-3^{AR+} than in PC-3^{AR-} cells. Transfections were performed with 0.5 ng of wtAR or AR-F671I expression vector and the ARR3-tk-luciferase reporter in the presence of 0.1 nM DHT. For each cell line, data was normalized as percent luciferase activity for wtAR in the presence of DHT and represent the mean (±SEM) of five individually transfected wells.

AR variants identified in prostate cancer or derived in in vivo studies may be masked by analysis in PC-3^{AR-} cells. This provides one possible explanation for the disparate functional importance of AR variants and coregulators reported in the literature [42,63,68].

The observation that PC-3^{AR+} cells have markedly increased levels of AR coregulator proteins compared to PC-3^{AR-} cells, including both AR coactivators (e.g., GRIP1, Zac1) and the AR corepressor, SMRT [51,52], provides a mechanism for both increased functional capacity of transfected AR in PC-3^{AR+} cells and for tighter regulation of AR function in the absence of ligand. This may also be reflected by the inability of low levels of endogenous AR to mediate either transactivation or a proliferative response in PC-3^{AR+} cells. Moreover, as transcriptional regulators such as GRIP1 or Zac1 interact with multiple signaling pathways, the increased expression of these factors in PC-3^{AR+} cells may, in part, mediate the greater proliferation rate of this cell line as well as the increased activity of transfected AR compared with PC-3^{AR-} cells.

Although AR is expressed throughout prostate cancer progression [1,2,57,59], prostate tumors exhibit remarkably heterogeneous AR expression compared to non-malignant glandular epithelium, with AR protein often detected in as little as 50% of tumor cells [69–72]. In both clinical prostate cancer and mouse xenograft models of the disease, castration results in a marked decrease in AR expression and tumor quiescence [73,74]. Tumor regrowth following a variably latency period is associated with re-expression of the AR to pre-castrate levels [73,74]. It is commonly thought that re-emergent prostate cancer is derived from AR positive cells in the untreated tumor that contain changes permissive for continued androgen signaling in the castrate environment [1,75]. Alternatively, AR negative cells in the primary tumor could survive androgen ablation and subsequently acquire permissive changes in androgen signaling pathways and reactivation of AR expression. In support of this notion, tumor cells in prostate cancer xenograft bearing mice that survive androgen ablation have a marked reduction in AR function but retain a poised transcription complex on the promoter of androgen responsive genes [76]. As reactivation of AR expression and AR responsive genes accompanies tumor progression in vivo, the two PC-3 sublines, which have marked differences in AR expression and function, may reflect different stages of tumor progression following androgen withdrawal.

In conclusion, it appears that prolonged cell culture of PC-3 cells has resulted in the acquisition of phenotypic changes and/or the selection of different cell populations with markedly altered capacity to respond to AR activation. The differences between PC-3^{AR-} and PC-3^{AR+} cells provide evidence that the androgen-signaling axis can be sensitized in prostate cancer cells that have little detectable AR protein, and may be instructive in understanding the evolution of a castrate-resistant phenotype. In particular, our studies suggest that further analysis of the two different PC-3 cell lines is warranted to better define the factors controlling AR signaling during prostate cancer progression. Importantly, our studies suggest that the widespread indiscriminate use of PC-3 cells in the literature (usually without documentation of AR status) may have contributed to conflicting or misleading results, especially in the context of evaluating AR function and the mechanisms of androgen responsiveness in prostate cancer cells.

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The role of the androgen receptor polyglutamine tract: structural and functional consequences of glutamine tract variation and evidence for an optimal repeat length

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ABSTRACT

The androgen receptor (AR) gene contains a polymorphic trinucleotide repeat region, $(CAG)_n$ in its amino-terminal transactivation domain (NTD) that encodes a polyglutamine (polyQ) tract in the receptor protein. While the length of the (CAG)n repeat ranges from 6-39 in healthy individuals, variations in repeat length both within and outside the normal range are associated with disease, including impaired spermatogenesis and Kennedy's disease, and with the risk of developing breast and prostate cancer. While an inverse relationship between poyQ tract length and transactivation potential of the receptor may result from altered recruitment of accessory proteins, the molecular mechanisms by which polyQ length modulates these effects has not been elucidated. In this study, we provide detailed characterization of a somatic AR gene mutation detected in a human prostate tumor that results in interruption of the polyQ tract by two non-consecutive leucine residues (AR-polyQ2L). Compared to wtAR, AR-polyQ2L exhibits disrupted inter-domain communication (N/C interaction) and an associated lower protein level, but paradoxically has markedly increased transactivation activity. Molecular modeling and the response to cofactors indicates that AR-polyQ2L provides a more stable platform for the recruitment of accessory proteins than wild-type AR. Analysis of the relationship between polyQ tract length and AR function revealed a critical size (Q16-Q29) for maintenance of N/C interaction. That up to 99% of AR alleles in different racial-ethnic groups encode a polyglutamine tract within this range suggests that positive evolutionary selection may act to preserve N/C interaction as an essential component of androgen induced AR signaling.

INTRODUCTION

Trinucleotide repeat sequences occur throughout the human genome where they expand and contract during replication in a dynamic process that gives rise to increased or decreased length (length polymorphisms) in subsequent generations (1,2). Whereas the majority of trinucleotide repeats are located in non-coding sequences of the genome and have no discernable effects, those located in coding regions can influence gene expression, modulate the structure and stability of intermediate mRNAs, and can alter the function of the resultant proteins (3). The longer the trinucleotide repeat the more predisposed it is to expansion, a phenomenon known as anticipation, and when they expand beyond a critical number in a given coding region it almost invariably results in disease (4-8).

The most common trinucleotide repeat sequence found in human genes is $(CAG)_n$, which predominantly give rise to polyglutamine (polyQ) tracts rather than polyserine or polyalanine when located in the coding sequence (9). There are currently more than 15 hereditary neurological diseases related to expansion of a polyQ region in a causative protein, including Huntington's disease, dentatorubral-pallidoluysian atrophy, Machado-Joseph disease/spinocerebellar ataxia type I, II, III, spinocerebellar ataxia VI and VII, myotonic dystrophy, spinal and bulbar muscular atrophy/Kennedy's disease and two fragile X syndromes (4,5,10,11).

The human androgen receptor (AR) gene contains a polymorphic CAG repeat within its coding sequence that gives rise to an uninterrupted polyQ tract in the amino-terminal transactivation domain (NTD) of the receptor (Fig. 1). The normal distribution of the AR-CAG microsatellite has been reported as 6-39 repeats with a

median of 19-20 in African American, 21-22 in White Caucasian, 22-23 in Asian and 23 in Hispanic populations studied (12-19). Expansion of the AR-CAG microsatellite to 40 or more repeats causes the rare, X-linked, adult onset, neurodegenerative disorder known as spinal and bulbar muscular atrophy/Kennedy's disease (20,21). In addition to progressive muscle weakness and atrophy due to loss of brain stem and spinal cord motor neurons, men with this disorder frequently present with symptoms of partial androgen insensitivity (i.e., gynecomastia and testicular atrophy), indicative of aberrant AR function (22,23). Conversely, several studies have demonstrated that AR-CAG repeat length is inversely correlated with the risk of developing prostate cancer, decreased age of onset and/or increased risk of advanced disease at diagnosis (15,18,24,25)(reviewed in (26)). In addition, AR-CAG repeat length has been implicated in the inherited form of androgen insensitivity (AIS), hereditary hearing impairment, schizophrenia, benign prostatic hyperplasia and the risk of developing prostate, breast and endometrial cancers (27-33).

Although variation in CAG repeat lengths does not affect the androgen-binding affinity of the encoded receptor in culture (34,35), there is an inverse relationship between AR transactivation capacity and polyQ length within the normal range (25,36-38). Structural changes in ARs with polyQ tracts of 40 or more residues, as in Kennedy's Disease, leads to the formation of insoluble protein aggregates that include the AR and a number of its coregulator proteins (39-41). The precise mechanisms leading to clinical and pathological development of symptoms associated with this aggregation in Kennedy's disease are not known (42). Moreover, how variations in polyQ length within the normal range affect AR function and influence the

development and progression of the non-neurological polyQ associated disorders has not been fully elucidated.

In addition to germline length polymorphisms, recent data suggest that the AR-CAG tract is a target for somatic alterations in clinical prostate cancer and animal models of the disease, including both contractions in repeat length and missense mutations (26,43-46). Of particular relevance to the present study is a dual somatic missense mutation within the AR-CAG repeat [$(CAG)_{22}CAA$ to $(CAG)_{12}CTG(CAG)_6CTGCAA$] (Fig. 1) detected in our previous studies of human prostate cancer (26) that results in the disruption of the polyQ tract by two leucine residues (AR-polyQ2L; Fig. 1). The main objectives of this study were to determine the consequences of the polyQ2L mutation for AR function in order to provide a molecular mechanism for its selection in human prostate cancer, and to better define the role of the polyQ tract in AR function.

RESULTS

AR-polyQ2L has increased activity but lower protein level compared to wtAR

The AR-polyQ2L variant was reconstructed in an AR expression vector and assayed for transactivation function by transient transfection of the AR negative human prostate cancer cell line, PC-3. Compared to wtAR, the AR-polyQ2L exhibited an approximate 2-fold greater activity than wtAR on the androgen responsive probasin promoter (ARR3-tk-luc) over a range of DHT concentrations (0.01-10nM) (Fig. 2A). Addition of a 1000-fold excess of the AR antagonist, hydroxyflutamide, blocked the DHT induced activity of wtAR and AR-polyQ2L with similar efficiency (data not shown). Immunoblot analysis of PC-3 cells transfected under similar conditions consistently demonstrated an approximate 40-50% lower level of AR-polyQ2L steady state protein compared to wtAR (Fig. 2B). When AR transactivation activity was corrected for this difference in protein level, AR-polyQ2L was up to 4-fold more active than wtAR (Fig. 2C). A similar augmented transactivation capacity of AR-polyQ2L compared to wtAR was observed with the androgen responsive prostate specific antigen (PSA) luciferase reporter gene (PSA540-luc; Fig. 2D). In this latter experiment, immunoblot analysis was performed directly on lysates used for analysis of transactivation function, confirming a lower steady state level of AR-polyQ2L protein in transfected cells. Independent analysis using the androgen responsive mouse mammary tumor virus chloramphenicol acetyl transferase reporter construct (MMTV-CAT) confirmed both the increased activity and lower protein levels of the AR-polyQ2L variant compared to wtAR (Fig. 2E, F).

Transfection of increasing amounts of wtAR expression vector with either the ARR3-tk-luc or the PSA540-luc reporter constructs resulted in a near linear increase in

protein (Fig. 3A) and transactivation activity (Fig. 3B). In addition, the affinity of AR-polyQ2L for DHT (0.29±0.07 nM) was not significantly different ($p>0.05$; t-test) to wtAR (0.23±0.04 nM) in transiently transfected COS-1 cells. Together, these data indicate that the higher activity of AR-polyQ2L compared to wtAR cannot be attributed to (i) non-specific inhibition of wtAR activity (squelching) due to higher protein levels compared to AR-polyQ2L, (ii) limiting quantities of the reporter construct or (iii) altered affinity of AR-polyQ2L for DHT.

Steady state AR RNA levels

Quantitative real-time PCR analysis demonstrated no significant difference in the relative level of wtAR or AR-polyQ2L RNA 4 or 24 hours after transfection with the AR expression constructs (Fig. 4A). In contrast, immunoblot analysis performed on lysates of the same transfected cells demonstrated significantly less (~40%) AR-polyQ2L protein at 24 hours than wtAR protein ($p=0.041$; Fig. 4B). This observation is consistent with previous studies demonstrating that AR CAG repeat length within the normal range does not affect AR expression or mRNA stability (25,34-37,47). These findings suggest that altered AR protein stability or turnover rather than different transcription rates or RNA stability is responsible for the decreased AR-polyQ2L protein level in transfected cells.

AR-polyQ2L exhibits reduced N/C interaction

Conformational changes induced by agonist binding to the AR facilitates a strong inter- or intra-molecular interaction between the ligand-dependent AF-2 surface in the LBD and an α-helix forming peptide, ^{23}FQNLF^{27}, in the NTD. This is referred to as the N/C interaction (48-50). Numerous AR coregulators, which contain similar

LxxLL-like α-helix peptides, compete with the ^{23}FQNLF^{27} peptide for the AF-2 surface following ligand binding (51,52). The N/C interaction is dependent on agonist ligands and has been suggested to be a necessary requirement for AR transactivation *in vivo* (53). As the polyQ region is located only 21 residues C-terminal to the ^{23}FQNLF^{27} peptide, we sought to determine whether the polyQ2L mutation alters the capacity of the AR to undergo an N/C interaction. The AR-polyQ2L NTD was significantly less capable of inducing reporter gene activity in the mammalian two-hybrid N/C assay compared with the wtAR-NTD at all concentrations of DHT examined (Fig. 5). Immunoblot analysis conducted on pooled lysates from the N/C assay detected an equivalent amount of wtAR-NTD and AR-polyQ2L-NTD protein (Fig. 5), indicating that the difference in activity in the N/C assay was not a result of altered levels of AR-polyQ2L NTD protein. Moreover, these data suggest that the polyQ2L disruption affects the level of AR protein only in the context of the full-length receptor (compare Figs. 2 & 6). As the N/C interaction has been well documented to protect the AR from degradation (49,54), a reduction in N/C provides a mechanism to explain the lower steady-state AR-polyQ2L protein levels compared to wtAR in the presence of ligand.

The polyQ2L disruption does not affect AF-5 mediated AR activation

Transcriptional activity of the wtAR is driven primarily by ligand induced synergy between activation domains AF-1 and AF-2 (55), while the activity of ARs truncated in the LBD prior to amino acid 708 is constitutive and mediated by the distinct NTD activation function, AF-5 (refer to Fig. 1) (56). Transfection of increasing amounts of vector expressing AR(1-707) results in increasing steady-state truncated AR protein (Fig. 6A) concomitant with increasing transactivation activity in the absence of

exogenous DHT (Fig. 6B). In contrast to observations with full-length AR-polyQ2L, there was no difference in the constitutive activity of truncated AR-polyQ2L compared to wtAR (Fig. 6C). Similar results were obtained for different amounts of transfected AR expression vector on both tk81-PB3 and PSA promoters (data not shown). These results indicate that the increased transcriptional activity of full-length polyQ2L is mediated by changes to the ligand-dependent AF-1/AF-2 transactivation pathway, and is therefore dependent on the presence of the LBD and ligand. Moreover, these results indicate that reduced N/C interaction with AR-polyQ2L-NTD compared to wtAR-NTD (refer to Fig. 5) is not due to altered intrinsic activity of the NTD in that assay, but rather to a defect in the ability of the polyQ2L NTD to interact with the LBD.

Structural consequences of the polyQ2L mutation

While reduced N/C interaction provides a mechanism for the lower steady-state level of AR-polyQ2L protein compared to wtAR, the polyQ2L mutation is distinct from other AR variants with N/C defects in that it has markedly enhanced transactivation function compared to wtAR (refer to Fig. 2). As it has recently been demonstrated that structural order within the AR-NTD is correlated with recruitment of the transcription machinery and receptor activity (57,58), we sought to assess whether the polyQ2L mutation alters AR-NTD structure. Secondary structure prediction algorithms (59) suggest that disruption of the polyQ tract by two leucine residues results in an extended alpha-helix along the repeat sequence (Fig. 7A). In a more rigorous approach, we utilized simulated annealing and molecular modeling to generate theoretical structures of a pure polyQ tract of 23 residues and an equal length glutamine tract containing the dual leucine interruption. These structures indicate that

the leucine residues have a substantial impact on the shape and flexibility of the repeat sequence, resulting in an overall increase in structural order (Fig. 7B).

Coactivation of AR-polyQ2L by GRIP1 and RAN

It has previously been demonstrated that the length of the polyQ tract is inversely related to the transactivation response of the AR to p160 coactivators such as GRIP1 (38) and to the AR coregulator, RAN (60). In this study, coexpression of either GRIP1 or RAN resulted in a greater increase in activity of AR-polyQ2L compared to wtAR (Fig. 8A,B). However, the fold increase in AR activity in the presence of each coregulator was the same (approximately 2-fold) for both wtAR and AR-polyQ2L (Fig. 8A,B). In addition, mammalian two-hybrid analysis did not detect any significant difference in the ability of wtAR or AR-polyQ2L to interact with either full length GRIP1 or the C-terminal GRIP1 fragment (aa 1122-1462) that mediates its interaction with the AR-NTD (Fig. 8C). While these data indicate that that altered interaction with endogenous GRIP1 or RAN *per se* is not responsible for increased transcriptional activity of AR-polyQ2L, it supports the concept that the polyQ2L NTD, possible due to enhanced structural order, is more amenable to signal enhancement by coregulators than the wtAR NTD.

polyQ length and AR function

Although it is well accepted that AR polyQ length is inversely related to the transactivation capacity of the receptor (25,35,38,47,60-62), only one other group has compared more than three polyQ alleles within the accepted normal range of 6-39 repeats (63). In that study, only a modest effect of polyQ length was observed. Here, we conducted multiple transfection experiments with 6 ARs containing different

polyQ allele lengths, namely 9, 16, 21, 26, 29 and 35 residues, conclusively demonstrating that there is a highly significant inverse relationship between transactivation and polyQ length within the normal range ($p<0.001$; Fig. 9A). There was on average a 1.7% decrease in activity for each additional glutamine repeat. Significantly however, the linear relationship did not apply to an AR with 9 glutamine residues, which had a transactivation activity lower than an AR with 16 repeats ($p<0.05$) ($p=0.48$; Fig. 9A).

To better define how the polyQ influences AR function, we created a series of constructs expressing the AR-NTD with different polyQ lengths as either a fusion with the DBD of GAL4 (Fig. 9B), or the transactivation domain of pVP16 (Fig. 9D). In contrast to full-length AR, the size of the polyQ did not affect transactivation capacity of the AR-NTD ($p=0.17$; Fig. 9C), or interaction of the NTD with the p160 coactivator, GRIP1 ($p=0.59$; Fig. 9E). In contrast, while there was no change in N/C interaction for AR with 16-29 glutamine residues, a polyQ allele length of 9 or 35 resulted in a highly significant decrease (approximately 50%) in the capacity of the AR to adopt the N/C interaction ($p<0.05$; Fig. 9F). Together, the above results suggest that polyQ length alters AR transactivation function directed through AF-1, and that short and long repeats markedly disrupt the N/C interaction. These observations are consistent with the analysis of the AR-polyQ2L mutation, where disruption of the repeat did not affect AF-5 mediated transactivation of the AR or NTD interaction with GRIP1, but markedly reduced N/C interaction (refer to Figs. 5-7).

The role of N/C interaction

To further analyze the role of N/C interaction in AR function, we utilized two previously characterized AR LBD mutations; the T875A variant identified from the LNCaP prostate cancer cell line that results in promiscuous activation of the receptor by non-androgenic ligands (64), and the E895Q mutation in the core of the AF2 surface, previously reported to inhibit the N/C interaction without altering ligand binding characteristics of the receptor (65). Whereas only DHT could effectively induce an N/C interaction with the wtAR LBD, DHT, progesterone and hydroxyflutamide could all induce a strong N/C interaction with the LNCaP variant (Fig. 10A). These results parallel the agonist ability of these ligands on wild-type and LNCaP ARs as previously reported (53). In contrast, the E895Q mutation eliminated the capacity of the AR to form an N/C interaction in the presence of any ligand examined (Fig. 10A). However, transactivation analysis clearly demonstrated that the E895Q AR variant retains up to 60% of wtAR activity in response to DHT on both simple and complex promoters (Fig. 10B). These characteristics have previously been demonstrated for AR AF2 core sequence mutations (50,66), suggesting that N/C interaction is not an absolute requirement of AR transactivation activity *per se*. Importantly, immunoblot analysis demonstrated a marked reduction in AR-E895Q protein level compared to wtAR (Fig. 10C), consistent with a role of N/C in preventing receptor degradation (49,54) and with the concomitant decrease in N/C interaction and steady-state protein levels observed for the polyQ2L AR variant.

AR-polyQ length distribution

In order to better understand the implications of the above results, we re-examined available AR polyQ length distribution data from control populations in the published

literature (14,24,67,68) (Fig. 11A). Firstly, as noted by Edwards et al in 1992 but little appreciated in the vast majority of subsequent studies, polyQ length is not distributed in a gaussian fashion, but exhibits a bimodal or more complex pattern (14) (Fig. 11A). While this was more apparent for African Americans, distinct peaks and troughs were observed in all ethnic groups. These peak patterns were observed in each of the individual reports from which this data was compiled. We then assessed polyQ length distribution in relation to maintenance of maximal N/C interaction determined above (ie. 16-29 repeats; Fig. 11B). Intriguingly, only 0.7% of polyQ lengths are outside this range in Asian populations (longer), while it rises to 3.4% in Caucasians (longer and shorter), 6.5% in Africans Americans (shorter) and 8.8% in Hispanic Americans (longer), which parallels the relative risk of prostate cancer for these different ethnic groups.

DISCUSSION

The AR-LBD is highly ordered, consisting of 11 alpha helices and 4 beta sheets arranged in a highly conserved tertiary structure (69,70). Conformational changes in the LBD bought about by high-affinity ligand binding results in the formation of the conserved hydrophobic AF2 surface (69), which is the docking site contended by AR coregulators and the ^{23}FQNLF^{27} motif in the AR-NTD (65,71). In contrast, chemical analysis has revealed that the AR-NTD has only a small number of predicted secondary structure elements (Fig. 1A), and adopts a disordered but flexible structure typical of transactivation domains (57,58). Nonetheless, these limited structural elements correspond to key sequences implicated in AR function [eg. the ^{23}FQNLF^{27} motif (71) and the core sequences of AF-1a and AF-1b (72) (Fig. 1A)], and have been hypothesised as critical for interaction of the NTD with coregulators and members of

the basal transcription machinery (57,58). In this study, the demonstration that increased secondary structure in AR-polyQ2L is associated with enhanced transactivation capacity of the receptor in response to ligand and to exogenous coregulators compared to wtAR, provides direct evidence for the relationship between structural order of the AR-NTD and transcriptional competence. Critically, our data supports a model whereby ARs with long polyQ tracts have reduced overall NTD order compared to those with shorter repeats, and are therefore less able to recruit coregulators and components of the transcription machinery. This provides a mechanism to explain how polyQ length within the normal range is inversely related to recruitment of AR coregulators and AR activity.

The reduction in N/C interaction and protein levels for the AR-polyQ2L variant compared to wtAR is analogous to observations for AR mutations identified in AIS and derived *in vitro* in the ^{23}FQNLF^{27} motif (49,71,73). However, AR-polyQ2L is distinct from those mutations in that it retains both the structure of the ^{23}FQNLF^{27} motif and the integrity of the LBD. Coregulators will therefore be capable of binding to the intact AF2 surface of AR-polyQ2L with reduced competition from the N/C interaction compared to wtAR (51). This provides an additional mechanism for enhanced transactivation capacity of AR-polyQ2L compared to wtAR, which may act in concert with effects associated with increased NTD order discussed above.

While the results of this study and others demonstrate that the N/C interaction is not essential for activity of the AR *per se*, it is highly indicative of a normal response of the receptor to physiological androgens and acts to stabilize the receptor following ligand binding (73). In this study, the observation that N/C interaction is only

sustained for polyQ lengths of between 16 and 29 residues, and can be disrupted within this range by mutation, provides the first clear evidence that the polyQ tract plays a mandatory role ensuring proper androgen induced function of the human AR. Maximizing N/C interaction would serve to exclude coregulators from the promiscuous AR AF-2 surface (51), and could therefore be an important means of maintaining the specificity of AR function. That approximately 95% of polyQ alleles across all ethnic groups within the normal population are between 16-29 residues, a range that would maintain maximum N/C interaction, strongly supports this hypothesis.

While CAG repeats can encode three distinct homopolymeric amino acid stretches [polyglutamine (CAG), polyserine (AGC) or polyalanine (GCA)], they are biased in coding regions for polyQ, suggesting a physiological role for glutamine over the alternative amino acids (9). polyQ tracts are found in diverse and critical proteins such as steroid receptors, nuclear receptor coactivators (AIB1), the TATA box binding protein subunit of transcription factor IID, CBP and IL-2 (3,74-77). The observations that more than 80% of human genes with a polyQ tract length of greater than 20 repeats are transcription factors, the corresponding regions from other mammalian species are statistically shorter or interrupted by other triplets, and that polyQ tracts are absent from prokaryotic transcription factors, suggests that polyQ regions may have evolved as regulatory elements involved in fine-tuning gene expression (3,78-80). In support of this, the positional context of polyQ tracts within transcription factors is a critical determinant of their transcriptional competence (9). A functional role for polyQ in protein function is also supported by evolutionary adaptations to the maintenance of polyQ tracts in particular proteins (74), and the observation that

sequences flanking polyQ regions have undergone nucleotide substitutions with much greater frequency (80). Importantly, the length of the polyQ tract in some proteins (eg. CBP) is absolutely conserved, suggesting that there are critical reasons for maintaining a particular repeat length in some cellular regulators (81). For the AR, the distribution of polyQ alleles in all ethnic populations examined was positively skewed (ie. towards longer repeats), which may reflect the bias of CAG repeats to expansion, a greater tolerance for longer alleles in maintaining AR function, or repeat length constraints (2,82). The distribution of AR polyQ alleles also exhibited a degree of biphasic distribution, depending on the particular racial-ethnic group considered which may reflect different founder effects in those populations.

On the basis of this study and the above data, we propose that polyQ tracts regulate protein function by serving as flexible spacers to separate regions of biological activity. In the context of the AR, maintenance of a polyQ of 16-29 residues would allow sufficient flexibility for the upstream ^{23}FQNLF^{27} motif to fold into the LBD (N/C interaction) while maintaining the capacity of the proximally located activation function (AF-1) to interact with coregulators and the transcription machinery. This provides a mechanism to explain how both increased and decreased polyQ allele length, and altered structure as observed for the polyQ2L mutation, can influence N/C interaction and receptor function. We further propose that the moderate reduction in AR activity with increasing length of the polyQ within the normal range is a tolerated consequence of maintaining greater receptor control. This mechanism may be distinct from how polyQ tracts expanded beyond 39 repeats cause Kennedy's disease, as recent studies suggest that the fundamental properties of polyQ tracts change when

they reach this length (42,83). However, in the absence of an N/C interaction, the expanded polyQ may be more vulnerable to aggregation.

Missense mutations identified in the AR-LBD in prostate cancer collocate to discrete regions of the receptor and have been well characterized, often resulting in receptors with increased promiscuity for activation by androgens and non-classical ligands (reviewed in (26,84-86)). This provides a mechanism for continued AR function following treatment of the disease by androgen ablation strategies, and suggests that collocation of mutations defines regions critical for normal AR signaling. While only a small number of studies have examined the AR-NTD for mutations in prostate cancer, we recently reported that they occur with higher frequency following androgen ablation, and predominantly collocate to either the polyQ tract or to a small region of the transactivation function, AF-5 (26,87). However, the functional consequences of the majority of these mutations are unknown. In this study, we have utilized the AR-polyQ2L variant to demonstrate a new mechanism whereby missense mutations identified in the AR-NTD in prostate cancer can exhibit a gain of function phenotype, thereby potentially contributing to disease progression.

The observation that normal AR function is sustained over a critical, but limited, range of polyQ lengths, could in part explain why analysis of AR CAG repeat length and risk of prostate cancer and other diseases has produced conflicting results. Our data suggest that changes in functionality for a receptor with a polyQ length either shorter or longer than the critical range of 16-29 residues could be a more important mediator of disease phenotype than a stepwise reduction in activity with increasing polyQ length across the entire range. In this model, ARs with a polyQ tract within the

critical range can be considered as functionally equivalent, while those with shorter or longer repeats as distinct. A consequence of this model is that division of samples by median AR-CAG repeat length, as performed in many studies, would bias for the null hypothesis. Therefore, reassessment of existing AR-CAG repeat data and defining the downstream effects of polyQ alterations is warranted to better understand the endocrine basis of AR related diseases and the assessment of risk in pre-symptomatic individuals.

MATERIALS AND METHODS

Cell Culture

COS-1, CV-1 and PC-3 cells were obtained from the American Type Culture Collection (ATCC; Rockville, MD) and maintained in RPMI 1640 medium (GIBCO, BRL, Melbourne, Australia) supplemented with 5% fetal bovine serum (FBS). Antibiotics were not used in culture medium.

Plasmid vectors and sequencing

DNA Sequencing: Manual sequencing in tumor samples was performed using the *fmol*™ DNA sequencing system (Promega Corporation, Madison, USA) as previously described (88). Plasmid integrity and the presence of the required base substitutions in AR variant plasmid constructs was confirmed by automated sequencing of each clone using AIB Big-Dye Terminators and an Applied Biosystems 3700 DNA analyzer (Foster City, CA, USA).

Reporter genes: The probasin (ARR3-tk-luc) and MMTV (MMTV-CAT) expression vectors have been used previously in our studies (38). pGL3-PSA540 consists of the androgen responsive regions of the PSA enhancer (nucleotides 502-1951) and promoter (nucleotides 4924-5784; Genbank accession #U37672) driving expression of the firefly luciferase gene. The pGK1 reporter used in mammalian two-hybrid assays consists of a minimal adenovirus E1b promoter and five tandem Gal4 response elements that drive luciferase gene expression (89).

AR-LBD mutants: LBD AR variant expression vectors were generated from the parental AR expression clone, pCMV-AR (90) using a PCR based megaprimer

method as previously described (91). Briefly, megaprimers were created by PCR, using pCMV-AR as template, with a downstream primer (5'-CCTCTAGAGTCGACCTGCAGG-3') spanning a HindIII site in the vector polylinker 3' to the coding sequence, and with a specific upstream mutagenic primers: (M-T778, 5'-AAGTCCCGGA**C**GTACAGCCAGTGTG-3'; T-A875, 5'-CATCAG**T**TC**G**CTTTTGACCTGCTA-3'; E-Q895, 5'-ATGATGGC**A**CAGATCATCTCTGTG-3') incorporating the required base mismatch (bold, underlined). Megaprimers were extracted from an agarose gel, purified, and used in individual PCR reactions with an upstream oligonucleotide (AR:HindIII-s; 5'-TGGAGATGAAGCTTCTGGGTGT-3') spanning the *HindIII* site (underlined) within the AR coding sequence. PCR products were digested with *HindIII* and cloned into pCMV-AR that had had the homologous wild-type sequence removed with the same enzyme.

PCMV-AR-polyQ2L was created using a variation of the megaprimer method described above. A pGEM-T™ (Promega Corporation, Madison, NY) clone enclosing the 413bp PCR product of the AR gene amplified from tumor p332 using oligonucleotides 1-1s and 1-1as (88) was digested with *BglI*. The resulting 220bp fragment spanning the mutant polyQ region and an upstream *Sma*I restriction site unique to the AR, was purified from an agarose gel and used as a megaprimer as described above with an antisense oligonucleotide (5-TGACACCCAGAAGCTTCATCTCCA-3') directed at a *Hind*III site (underlined) located in the AR coding sequence and unique to pCMV-AR. The resulting fragment was purified by gel extraction, digested with *Sma*I and *Hind*III and cloned into

pCMV-AR(ms-) in which a wild-type AR fragment had been removed with the same enzymes, yielding pCMV-AR-polyQ2L.

AR-polyQn expression vectors: To construct pcDNA-AR(CAG)$_n$ vectors encoding ARs with different poly-Q lengths, NheI-AflII fragments were PCR amplified from genomic DNA samples of males with known CAG repeat lengths (sense primer, 5'-GAAGTTTCCTTCTCTGGAGCTTCC-3'; antisense primer, 5-'GTTGCATGGTGCTGGCCTCGCT-3') and inserted into pcDNA-hAR digested with the corresponding restriction enzymes.

Truncated AR expression vectors: pCMV-AR-trunc707, which expresses an AR truncated following amino acid 707, was generated as follows by PCR. An AR fragment was amplified using the AR-HindIII-s oligonucleotide (listed above) and an antisense oligonucleotide primer (Trunc707-as; TGCATCTAGA**TCA**CTCTCCCAGTTCATTGAGGCTAGA), which is directed at the AR coding sequence upstream of codon 708 and incorporates a STOP codon (bold) and an *XbaI* restriction site (underlined). PCR product was digested with *HindIII* and *XbaI* and cloned into the analogous sites of pCMV-AR to yield pCMV-AR-trunc707. The AR-polyQ2L-trunc707 expression vector was derived by transferring the EagI-BstEII fragment of pCMV-AR-polyQ2L to pCMV-AR-trunc707 cut with the same enzymes.

AR mammalian two-hybrid vectors: pM-AR(LBD) contains AR residues 644-917 cloned in-frame with the GAL4 DNA binding domain of pM (BD Biosciences Clontech, Palo Alto, CA) and is described elsewhere (92). pVP16-AR(NTD), which

encompasses AR amino-acid residues 1-538, was generated by PCR using a sense oligonucleotide (AR-1-s; AGCTGAATTCATGGAAGTGCAGTTAGGGCTG) incorporating a *EcoR1* site (underlined) with an antisense oligonucleotide (AR-538-as; ATGCTCTAGA**TCA**GGCTGTGAAGAGAGTGTGCCA) that incorporates an *XbaI* site (underlined) and a STOP codon (bold). The PCR product was digested with *EcoR1* and *XbaI* and cloned into the analogous sites of pVP16 (Clontech). The AR-polyQ2L mutation and different (CAG)n lengths were transferred from the pCMVAR-polyQ2L or pcDNA-AR(CAG)n expressions vector using *NarI* and *BfrI* (358bp fragment) to the homologous sites of pVP16-AR(NTD). The AR-LBD mutations (M778T, T875A, E895Q) were transferred from pCMV-AR expression vectors using *BseAI* and *SfuI* (183bp fragment) to the homologous sites of pM-AR(LBD).

Transactivaiton assays

Transfection and analysis of luciferase activity in 96-well plates: Cells were seeded in 96-well plates in RPMI1640 medium containing 5% fetal calf serum (FCS) at a density of 5000-20000 cells/wells and incubated for 16 hours. Immediately prior to transfection, cells were washed with serum free, phenol red free RPMI-1640 medium. Transfection was carried out for four hours in the same medium with the appropriate vectors (see below) mixed with LipofectAMINE™ (GIBCO-BRL) (0.4μl for each 96 well) according to the manufacturer's protocol. Four hours after transfection, the reaction mix was removed, and cells overlaid with phenol red free RPMI-1640) containing 5% dextran coated charcoal stripped foetal bovine serum (DCC-FBS) supplemented with 0.01-100nM of the appropriate steroid (stock solution dissolved in ethanol added to medium at no greater than 1μl/ml) or ethanol carrier alone. Cells

were incubated for 24-36 hours and then harvested directly from plates by adding 50μl Passive Lysis Buffer (Promega) per well and incubating for 20 minutes with aggitation. To assay for luciferase activity, 25ml from each well was transferred to an optical plate and assayed immediately for reporter and control gene activities with the Dual-Luciferase™ Reporter Gene Assay (Promega, Sydney, Australia) using a plate reading luminometer (Top Count™, Packard, Mount Waverley, ACT). The remaining sample was then frozen at –70°C for immunoblot analysis.

Analysis of AR transactivation capacity in 96 well-plates: For assays of AR transactivation, the plasmid mix included 0-10ng of wtAR or AR variant expression vectors [pCMV-AR, pcDNA-AR(CAG)n] and 100ng of the appropriate androgen responsive reporter construct (ARR3-tk-luc, MMTV-luc, pGL3-PSA540-luc). The effect of cofactors on AR activity was examined by transfection with a plasmid mix containing 1ng of pCMV-AR, 100ng of the appropriate androgen responsive reporter construct (ARR3-tk-luc, MMTV-luc or pGL3-PSA540-luc), and 50ng of the appropriate coactivator expression vector. Transfection mixes were carefully balanced with respect to the molar ratio of expression vectors (using the appropriate empty vector) and total plasmid [using the promoter-less vector, pCAT-basic or the prokaryotic vector, pBS-sk(-)] to control for any spurious activity due to squelching of basal transcription factors or secondary co-regulatory proteins.

Mammalian two-hybrid assays in 96 well plates: For mammalian two-hybrid assays, COS-1 cells (15,000 cells/well) were cotransfected in 96 well plates, as described above, with an equal molar amount of both pM and pVP16 vectors containing the appropriate genes or fragments of interest fused in frame with the GAL4-DBD or

VP16 activation domain respectively (maximum 15ng of each vector), and 25ng of the pGK1 reporter. The total molar amount of each parental expression vector was kept constant in each well by the addition of empty pM of VP16 vector. In addition, the total amount of DNA was kept constant for each well by adding an appropriate amount of either the promoter-less vector, pCAT-basic or prokaryotic vector, pBS-sk(-). Following transfection, cells were treated for 30 hours in phenol-red free RPMI1640 supplemented with the appropriate steroids. Luciferase activity was determined in cell lysates as detailed above.

CAT assays: For assays of transcriptional activity using chloramphenicol acetyl transferase (CAT), 10^6 PC-3 cells were seeded into 60 mm dishes 24 hours prior to transfection. Transfection of was performed using LipofectAMINE reagent (20μl per dish) according to the manufacturer's protocol. Cells were co-transfected with pCMV-AR or pCMV-AR-polyQ2L (50ng) and with the ARR_3-tk-CAT reporter construct (2.0μg), which is composed of a minimal thymidine kinase (tk) promoter under the control of three identical fragments of the rat probasin promoter (nucleotides -244 to -96). For coactivator analysis, cells were additionally co-transfected with pcDNA3.1-AIB1 (93) or an appropriate amount of empty pcDNA3.1(+) vector. Total DNA in each transfection was kept constant. After transfection, cells were grown in RPMI media (without phenol red) containing 10% charcoal/dextran-stripped FBS (Gemini Bio Products, Calabasas, CA) for 48 hours in the presence or absence of DHT for the last 24 hours. Whole-cell extracts were prepared in 0.25 M Tris-HCl (pH 8.0) by repeated freezing and thawing. Total cellular protein was measured using the Bio-Rad (Hercules, CA) Protein Assay Kit and CAT

assays were performed using the Quant-T-CAT Kit (Amersham-Pharmacia Biotech, Piscataway, NJ). CAT activities were corrected for total cellular protein (cpm/OD_{595}).

Immunoblot analysis

Immunoblot analysis was performed on lysates of transfected cells by three methods. (i) In the Dame Roma Mitchell Cancer Research Laboratories, cells transfected in 100mm dishes with pCMV-hAR3.1 or pCMV-hAR-polyQ-L_2 were harvested into 1.2 ml ice cold cytosol buffer (10 mM Tris.HCl, 1.0 mM EDTA, 10% (w/v) glycerol, 10 mM sodium molybdate, 0.2 mM dithiothreitol, 1.0 mM phenylmethylsulfonylfluoride; pH 7.2). Soluble cytosol fractions were prepared by centrifugation at 100,000g for 30 minutes, and the protein concentration determined using the Bradford protein assay kit (BioRad, Regents Park, NSW, Australia). Total cellular protein (20mg) was electrophoresed on a 6% SDS-polyacrylamide gel, transferred to Hybond-C membrane (Amersham) and immunostained using an affinity purified rabbit polyclonal antibody (U402; 1:200 dilution) specific for the N-terminal 21 amino acids of the AR (94) and a mouse monoclonal cytokeratin 8 antibody (Sigma; 1:1000 dilution). Immunoreactivity was detected using horse-radish peroxidase conjugated sheep anti-rabbit IgG (Silenus; 1:2000) and horse-radish peroxidase conjugated goat anti-mouse IgG (Dako Corporation, Carpinteria, USA; 1:4000), respectively and visualised using ECL Western blotting reagents (Amersham). AR and cytokeratin 8 protein levels were determined from immunoblots using an Imaging Densitometer (BioRad). (ii) In immunoblot analyses conducted at the Norris Comprehensive Cancer Centre in parallel to CAT assays, PC-3 cells were transfected in 60mm culture dishes and treated as detailed in the appropriate section above. Transfected cells were harvested in 100 µl RIPA buffer [10 mM sodium

phosphate, 2 mM EDTA, 150 mM NaCl, 50 mM NaF, 0.1% SDS, 1% Nonidet NP-40, 1% sodium deoxycholate, 0.2 mM Na3VO4, (pH 7.2)] that contained mammalian protease inhibitors. Total cellular protein was measured using the Bio-Rad Protein Assay Kit and equal amounts of each extract were analyzed by SDS-PAGE. Proteins were transferred to Hybond-P membrane (Amersham-Pharmacia Biotech) and probed with rabbit polyclonal anti-AR antibody N20 (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 μg/ml, or with mouse monoclonal anti-cytokeratin peptide 8 (CK8) antibody (Sigma, St. Louis, MO) at 10 μg/ml. Horseradish peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology) at 80 ng/ml or goat anti-mouse IgG (Bio-Rad) at 1:3000 dilution was used as the secondary antibody. Proteins were visualized by membrane treatment with Luminol Reagent (Santa Cruz) and exposure to Hyperfilm ECL (Amersham-Pharmacia Biotech). Autoradiograms were analyzed by quantitative scanning densitometry using a Bio-Rad Model GS-710 Imaging Densitometer. (iii) Immunoblots were also performed on lysates pooled from replica samples in 96-well plates following transactivation or mammalian two-hybrid assays. Immunoblots on these samples were performed a detailed in (i) above.

Dissociation rate

Dissociation of [1,2,4,5,6,7-^{3}H]-5α-dihydrotestosterone ([^{3}H]-DHT; Amersham) from wtAR and AR variants was examined in monolayer cultures of COS-1 cells (10^6 cells per 10cm culture dish) as previously described (95). Cells were transiently transfected with 20μg of wtAR or AR variant expression vectors using DEAE Dextran (0.5mg/ml; Sigma, St. Louis, MO; 30 minutes at 37^0C), and subsequently cultured in Dulbecco's modified Eagle's medium (Gibco-BRL) containing 5% charcoal stripped fetal bovine serum for 48 hours. Parallel cultures were pre-incubated in Dulbecco's

modified Eagle's medium (DMEM; Gibco-BRL) containing 5% charcoal stripped fetal bovine serum and 3 nM [^{3}H]-DHT in the presence or absence of a 200-fold excess of unlabelled DHT for 1h at 37^{0}C. Medium was replaced with DMEM containing 5% charcoal stripped fetal bovine serum and 0.5 μmol/L unlabelled DHT. Cyclohexamide (500 μmol/L) was included to minimize synthesis of new AR.

Affinity

Assays for affinity of AR for DHT were performed as previously described (90,95,96). Briefly, COS-1 cells (10^6 cells per 10cm culture dish were transiently transfected with 20μg of wtAR or AR-polyQ2L expression vectors using DEAE Dextran (Sigma, St. Louis, MO) and cultured in medium containing 5% charcoal stripped fetal bovine serum for 48 hours. Cells were harvested into ice cold cytosol buffer and soluble fractions prepared by centrifugation at 100,000g for 30 minutes. Fractions were incubated with 0.1-6.0 nM [^{3}H]-DHT for 16 hours at 4^{0}C before excess steroid was removed using dextran coated charcoal and centrifugation. Specific activity in fractions was determined by liquid scintillation and the affinity calculated using Scatchard plot analysis and linear regression.

Quantitative real-time PCR for RNA and plasmid DNA in transfected cells

RNA: PC-3 cells were plated at a density of $7.5x10^5$ cells per well in 6 well culture dishes and allowed to adhere overnight. Individual wells were transfected with AR expression vectors, pCMV-AR and pCMV-AR-polyQ2L (250ng) using 20μl LipofectAMINE according to the manufacturers protocol. Four hours after transfection, cells were washed with normal media, and overlaid with phenol red-free RPMI containing 5% charcoal stripped-FBS and supplemented with 1nM DHT. Four

and 24 hours later, cells were harvested and total RNA extracted using the RNeasy Mini Kit (Qiagen) according to the manufacturers instructions. RNA integrity was assured by gel electrophoresis on a 1% agarose-MOPS gel containing 18% formaldehyde. A 15μg portion of each RNA preparation was treated in a 50μl reaction containing 10U DNaseI, 40U RNasin, and 5mM DTT (final concentration) in 1xDNAse buffer at 37^0C for 30 minutes. RNA was extracted by vortexting with 50μl phenol:chloroform:isoamyalcohol (P:C:I) and centrifugation at maximum speed for 5 minutes at room temperature. The aqueous layer was transferred to a new tube and the P:C:I mix back extracted with an additional 50μl of DEPC treated H_20, which was collected as above and pooled with the first fraction. RNA was precipitated by the addition of 0.1 volumes of 3M sodium acetate (pH5.2), 2.5 volumes of 100% EtOH and 1μl glycogen (20mg/ml) at -70^0C for 2 hours. RNA was collected by centrifugation at maximum speed for 20 mins at 4^0C, washed with 500μl of 70% EtOH, and air dried for ~20 mins until EtOH evaporated. RNA pellet was allowed to dissolve for more than one hour in 12.5ml sterile water. RNA concentration was determined by spectrophotometry, and the integrity of RNA preparations by electrophoresis on a 1% agarose-MOPS gel containing 18% formaldehyde (Appendix A5.4). RNA was reverse transcribed in a reaction containing 500ng DNase treated RNA, 100ng random hexamers and 200U SuperscriptII™ reverse transcriptase (Gibco-BRL) according to manufacturers instructions. A duplicate aliquot of each RNA preparation was treated similarly, but without the addition of the reverse transcriptase enzyme, and were used to confirm that no contaminating genomic DNA was present. The integrity of each cDNA preparation was determined by PCR for β-actin using specific primers (BAs, 5'-GCCAACACAGTGCTGTCTGG-3'; BAas, TACTCCTGCTTGCTGATCCA-3').

DNA: A second aliquot of the primary RNA preparation was treated with 2U DNAse free RNAse A (Boehringer Manheim) and extracted similarly to yield sheared genomic DNA and AR vector DNA from transfected cells.

QRPCR reactions: To determine relative RNA levels, reactions were set up with 10µl of 2xSYBR Green PCR Mastermix (Applied Biosystems), 8µl of a 1/100 dilution of cDNA and 5pmol of specific sense and antisense primers for either β-actin (as above) or AR (AR2190s, 5'- AGCCATTGAGCCAGGTGTAGTGTG-3'; AR2420as, 5'- GTGAAGGATCGCCAGCCCAT-3'). QRPCR was performed on a Rotor-Gene 2000 (Corbett Research, Mortlake NSW) instrument using the following parameters: incubation at 94^0C for 10 min; 50 cycles of 94^0C for 15 sec, 60^0C for 15 sec and 72^0C for 30 sec. Instrument melt curve analysis and gel electrophoresis were used to assure amplification of a single product. The relative expression level of wtAR and AR-polyQ2L was determined by normalisation of cycle threshold values to those of β-actin for each sample using Rotor-Gene software (Version 4.4 Build 1). To determine the level of transfected AR vector, analogous reactions were set up with the same primers using the DNA and vector preparations described above. As the AR primers span the approximately 13kb intron 4 of the AR gene, they are specific for the vector DNA in these samples. β-actin primers provide the same fragment for DNA as for RNA above.

Modelling of the AR-polyQ tract

Molecular modelling of the AR polyQ tract was performed by constructing an alpha-helical polyQ stretch as a free peptide and subjected it to simulated annealing with

molecular dynamics using the program Biomer (http://www.scripps.edu/~nwhite/B). This simulation was repeated using a polyQ peptide containing the two leucine substitutions. Both simulations treated the polyQ tract as a free peptide rather than a constrained peptide within a larger assembly. Secondary structure predictions were performed on sequences of at least 50 residues in length using the NNPredict program of Kneller (1990) available online at <http://www.cmparm.ucsf.edu/~nomi/nnpredict.html>.

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FIGURE LEGENDS

Figure 1. Structure of the human androgen receptor and identification of a novel mutation in the AR-CAG repeat in an advanced human prostate tumor. Schematic representation of the AR showing the amino-terminal (NTD), DNA binding (DBD) and ligand binding (LBD) domains, activation functions [AF-1, AF-2, AF-5], secondary structure elements (α-helices, h/H) determined by chemical (58) or crystal structure (69,70) analysis, and recognized subdomain structures within the NTD including polymorphic repeat sequences (polyglutamine, polyQ; polyproline, polyP; polyglycine, polyG), core regions of AF-1 [AF-1(a) and AF-1(b)] and motifs responsible for inter-domain interactions (i.e. ^{23}FXXLF^{27} and ^{432}WXXLF^{436}). Truncation of the AR in the LBD at amino acid 707 (indicated) results in a constitutively active receptor. AR-polyQ tract lengths in the normal population vary from 6-39 residues distributed about a mean of between 19-23 residues depending on the ethnic group analyzed. Sequencing traces show the polyQ coding CAG repeat of a representative control sample and from prostate tumor sample p332. Two A-T base substitutions identified within the CAG repeat of a subpopulation of sequences derived from sample p332 results in interruption of the encoded polyglutamine (polyQ) repeat of the receptor with two leucine (L) residues as indicated.

Figure 2. Transactivation capacity and protein levels of wtAR and AR-polyQ2L. (**A**) Transactivation capacity of wtAR and AR-polyQ2L determined in transiently transfected PC-3 cells on the probasin (ARR3-tk-luc) promoter. Data is expressed as a percentage of the luciferase activity induced by wtAR in the presence of 1nM DHT, and represents the mean (±sem) of 8 independently transfected wells. (**B**) AR and

cytokeratin 8 (α-CK8) immunoblot performed at the University of Adelaide using 20mg of total cellular protein derived from PC-3 cells transfected with either wtAR or AR-polyQ2L expression vectors. Average steady-state AR level determined from densitometric analysis of independent immunoblots and corrected for α-CK8 is presented relative to wtAR. (**C**) Transactivation capacity of wtAR and AR-polyQ2L from A corrected for relative AR protein expression determined by densitometric analysis of immunoblots in B. (**D**) Relative transactivation capacity of wtAR and AR-polyQ2L in transiently transfected PC-3 cells on the pGL3-PSA540-luc promoter. Transactivation data was corrected for protein expression (inset), determined in the same transfected samples by immunoblot analysis, and represents the mean (±sem) of 8 independently transfected wells. Average steady-state AR level determined from immunoblot analyses is presented relative to wtAR. (**E**) Investigation of AR and cytokeratin 8 (α-CK) protein levels by immunoblot analysis performed at the Norris Comprehensive Cancer Centre as detailed in B. Average steady-state AR level determined from immunoblot analyses is presented relative to wtAR. (**F**) Relative transactivation capacity of wtAR and AR-polyQ2L on the MMTV promoter (MMTV-CAT) in PC-3 cells. Data was corrected for the average stead-state AR level determined from immunoblots as detailed above, and represents the mean (±sd) of 3 independent transfection experiments.

Figure 3. AR transactivation activity on the PSA540-luc and ARR3-tk-luc reporter genes and AR steady-state protein levels in PC-3 cells transfected with increasing amounts of wtAR expression vector. (**A**) PC-3 cells were transfected with 0.5-10ng of wtAR expression vector per well in 96 well plates as indicated, and with either the ARR3-tk-luc or PSA540-luc promoters. Transactivation data on each

promoter is expressed as a percentage of the luciferase activity in cells transfected with 10ng of wtAR expression vector in the presence of 1nM DHT and represents the mean (±sem) of 8 independently transfected wells. (**B**) Immunoblot analysis of AR and β-actin in lysates of PC-3 cells transfected with 0.5-10ng of AR expression vector per 15,000 cells (as indicated). AR steady-state levels determined from densitometric analysis of immunoblots is presented relative to β-actin in the same sample.

Figure 4. Relative wtAR and AR-polyQ2L mRNA and protein levels. PC-3 cells (1 million cells/well) in 6-well plates were transfected with wtAR or AR-polyQ2L expression vectors (6 wells each) and incubated with 10nM DHT. At the indicated timepoints, cells from each well were divided for protein and mRNA analysis. (**A**) Quantitative real-time PCR analysis for wtAR and AR-polyQ2L mRNA. Results represent the mean (±sem) mRNA level from the 6 independently transfected wells corrected for transfected plasmid levels also determined by QRPCR in the same samples as detailed in Materials and Methods. (**B**) AR immunoblot analysis using 20mg of total cellular protein performed on transfected samples used in A. Densitometric analysis of immunoblots was used to generate the graph, which represents the mean (±sem) AR level from the six independently transfected wells for each receptor. The level of AR-polyQ2L protein was significantly lower than wtAR (*$p<0.05$; Mann-Whitney U test).

Figure 5. The AR-polyQ2L mutation markedly inhibits the AR N/C interaction. Mammalian two-hybrid analysis of AR N/C interaction in COS1 cells (20,000 cells/well) transfected in 96-well plates with an equal molar ratio (maximum 15ng) of the indicated plasmids and 25ng of the pGK1-luc reporter construct, and incubated for

30 hours in the presence or absence of different concentrations of DHT as indicated. Data is shown as relative light units (RLU) and represents the mean (±sem) of 4 independently transfected wells. The N/C interaction was significantly lower for AR-polyQ2L NTD than for the wild-type NTD at most ligand concentrations (*$p<0.05$, Mann-Whitney U test). Immunoblot analysis of AR-NTD in pooled lysates from transfected wells in A as indicated. There was no difference in the steady-state protein levels of wtAR-NTD and AR-polyQ2L-NTD in transfected cells as determined from densitometric analysis of the immunoblot ($p>0.05$, t-test).

Figure 6. The polyQ2L mutation does not influence AR transactivation capacity directed by AF-5. (**A**) Immunoblot analysis of AR and β-actin in lysates of PC-3 cells transfected with 0.5-10ng of pCMV-AR(1-707) expression per 15,000 cells (as indicated). AR steady-state levels determined from densitometric analysis of immunoblots is presented relative to β-actin in the same sample. (**B**) PC-3 cells (15,000 cells/well) were transfected with 0.1-10ng of pCMV-AR(1-707) expression vector per well in 96 well plates (as indicated) and with the ARR3-tk-luc promoter. Transactivation data is expressed as a percentage of the luciferase activity in cells transfected with 10ng of pCMV-AR(1-707) expression vector and represents the mean (±sem) of 8 independently transfected wells. (**C**) No difference in the transactivation capacity of wtAR(1-707) and AR-polyQ2L(1-707) in PC-3 cells transiently transfected with 1ng of AR expression vector and the ARR3-tk-Luc promoter, as detailed in B, was observed ($p>0.05$, t-test). Data represents the mean (±sem) of 8 independently transfected wells.

Figure 7. Structural prediction and modeling of the AR polyQ tract. (**A**) The NNpredict algorithm of Kneller *et al* (59) was used to assess the effect of the polyQ2L AR mutation on putative secondary structural elements in the vicinity of the polyQ. H represents an α-helix. (**B**) An alpha-helical polyQ stretch representing wtAR, constructed as a free peptide, was subjected to simulated annealing with molecular dynamics using the program Biomer (http://www.scripps.edu/~nwhite/B). The simulation was then repeated for polyQ containing leucine substitutions as found in the AR-polyQ2L variant. Both simulations treated the polyQ tract as a free peptide rather than a constrained peptide of a larger assembly. This model confirms that the leucine residues have a substantial impact on the shape of the theoretical α-helical structure of the polyQ region.

Figure 8. Response of wtAR and AR-polyQ2L to coactivators. (**A**) The effect of the p160 coactivator, AIB1 on transactivation capacity of wtAR and the AR-polyQ2L variant was assessed on the MMTV-CAT reporter construct in transfected PC-3 cells as detailed in Materials and Methods. Data is expressed relative to the activity of wtAR in the presence of 1nM DHT and represents the mean (±sd) of three transfections. (**B**) The effect of the NTD coactivator, RAN on transactivation capacity of wtAR and the AR-polyQ2L variant was assessed on the ARR3-tk-luc reporter gene as detailed in Materials and Methods. Data is expressed relative to the activity of wtAR in the presence of 1nM DHT and represents the mean (±sem) of six transfections. (**C**) The mammalian two-hybrid assay was used to assess the effect of the polyQ2L AR mutation on the interaction of the NTD with the p160 coactivator, GRIP1. COS1 cells (20,000 cells/well) were transfected in 96-well plates with an equal molar ratio (maximum 15ng) of the indicated plasmids and 25ng of the pGK1

reporter construct and incubated for 30 hours in the absence of exogenous ligand. Data is shown as relative light units (RLU) and represents the mean (±sem) of 4 independently transfected wells. There was no significant difference in the ability of wtAR or polyQ2L NTD to interact with full-length GRIP1 or the GRIP1 fragment (aa1122-1462) known to interact with the AR-NTD ($p>0.05$, Mann-Whitney U test).

Figure 9. Role of the polyQ length in AR transactivation and N/C interaction. (**A**) Transactivation capacity of wtAR with different polyQ repeat lengths. PC-3 cells in 96 well plates (10,000 cells/well) were transfected with 0.5ng of AR expression plasmid containing different CAG repeat lengths (pCDNA-AR) and 100ng of the MMTV-luc reporter gene. Following transfection, cells were incubated for 30 hours in the presence of 1nM DHT as indicated. Results represent the amalgamated data (±sem) of 6 independent experiments each containing 7 individually transfected wells (ie. 42 datapoints). The decrease in activity for increasing polyQ repeat length was highly significant ($p<0.001$, ANOVA). (**B**) Immunoblot analysis for AR and β-actin on lysates of COS-1 cells transfected in six well dishes with pM-AR expression clones containing different CAG repeat lengths. (**C**) Transactivation capacity of the AR-NTD with different polyQ repeat lengths determined in COS-1 cells transfected with pM-AR expression clones and the pGK1 reporter corrected for protein level of each AR construct. There was no significant trend in AR-NTD activity with increasing polyQ repeat length ($p=0.17$, ANOVA). (**D**) Immunoblot analysis for AR and β-actin on lysates of COS-1 cells transfected in six well dishes with pVP16-AR expression clones containing different CAG repeat lengths. (**E**) Mammalian two-hybrid analysis for interaction of AR containing different polyQ lengths and the p160 coactivator, GRIP1, was performed as detailed in Figure 6. Data is shown as relative

light units (RLU) and represents the mean (±sem) of 6 independently transfected wells corrected for protein level of each AR construct. There was no significant trend in AR-NTD/p160 interaction with increasing polyQ repeat length ($p=0.59$, ANOVA). (**F**) Mammalian two-hybrid analysis for N/C interaction with AR containing different polyQ lengths performed as detailed in Figure 6. Data is shown as relative light units (RLU) and represents the mean (±sem) of 6 independently transfected wells corrected for protein level of each AR construct. While there was a highly significant effect of polyQ length on N/C interaction for the series ($p<0.001$, ANOVA), a significant change in N/C interaction was only observed between 9-16 and 29-35 repeats (*$p<0.05$, t-test).

Figure 10. The effect of AR-LBD mutations on transactivation capacity and N/C interaction. (**A**) AR N/C interaction for the LBD AR variants E895Q and T875A (LNCaP AR variant) was compared with the wild-type AR LBD using the mammalian two-hybrid system as detailed in Figure 6. Data is shown as relative light units (RLU) and represents the mean (±sem) of 4 independently transfected wells. (**B**). Transactivation capacity of wtAR and the AR-E895Q variant on two promoters. PC-3 cells in 96 well plates (10,000 cells/well) were transfected with 0.5ng AR expression plasmid (pCDNA-AR) and 100ng of either the AR responsive probasin (ARR3-tk-luc) or PSA (pGL3-PSA540-luc) reporter. Following transfection, cells were incubated for 30 hours in the presence or absence of 1nM DHT as indicated. Results represent the mean (±sem) of 6 individually transfected wells. (**C**). Immunoblot analysis of wtAR and the E895Q AR variant in transfected PC-3 cells.

Figure 11. Frequency distribution of polyQ lengths in different racial-ethnic groups. A. Amalgamation of AR polyQ length frequency data from control populations in the published literature (14,24,67,68). **B**. Parameters calculated from amalgamated data presented in A.

FIGURE 1

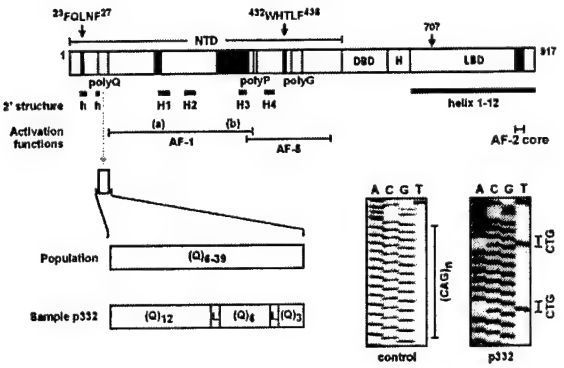


FIGURE 2

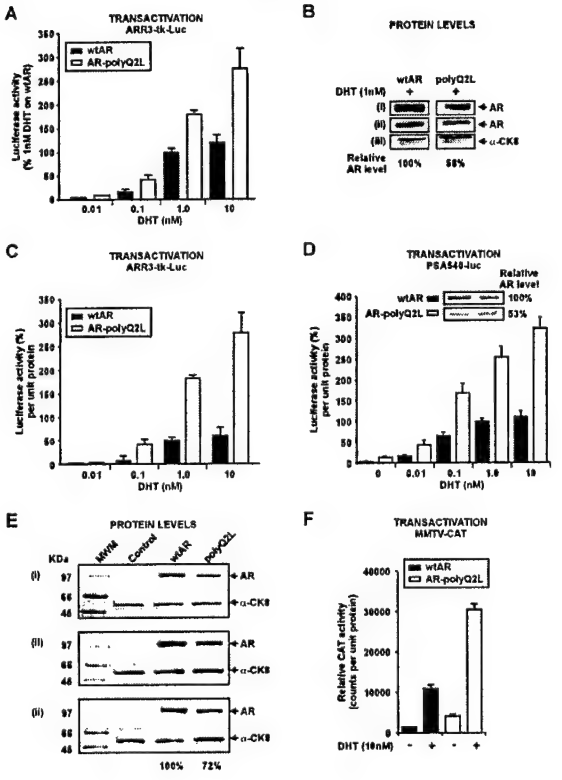


FIGURE 3

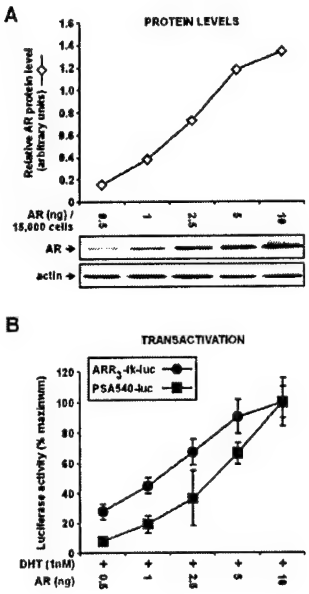


FIGURE 4

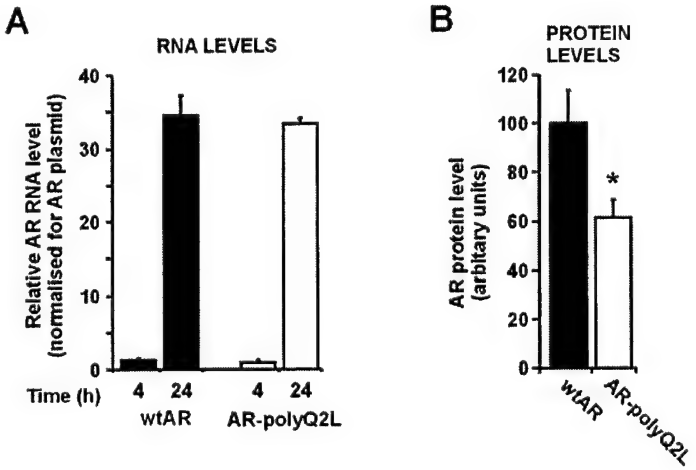


FIGURE 5

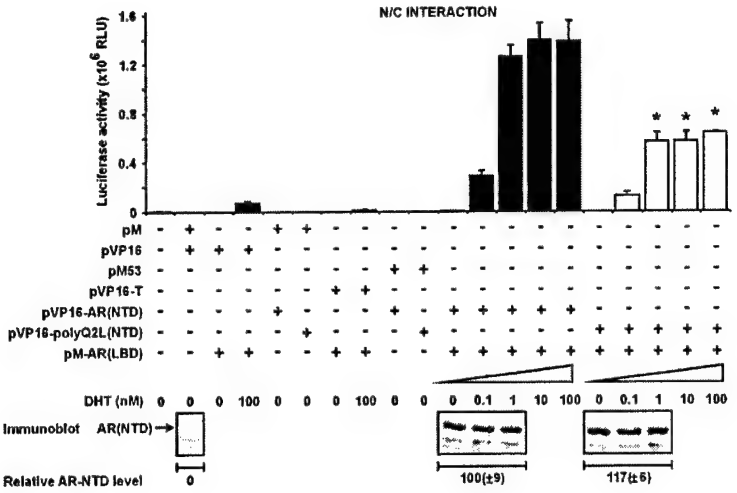


FIGURE 6

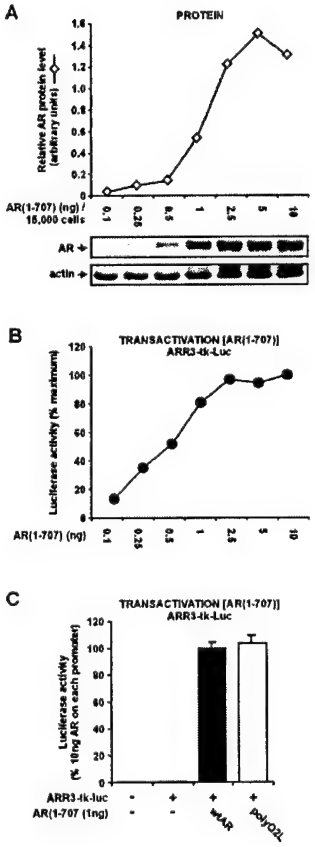


FIGURE 7

A

```
wtAR 49-82       ppgasllllqqqqqqqqqqqqqqqqqqqqqqqqet
                 -----HHHHHH----------------------
polyQ2L 49-82    ppgasllllqqqqqqqqqqqqlqqqqqqlqqqet
                 -----HHHHHH------HH-HHHHHHHH------
```

B

FIGURE 8

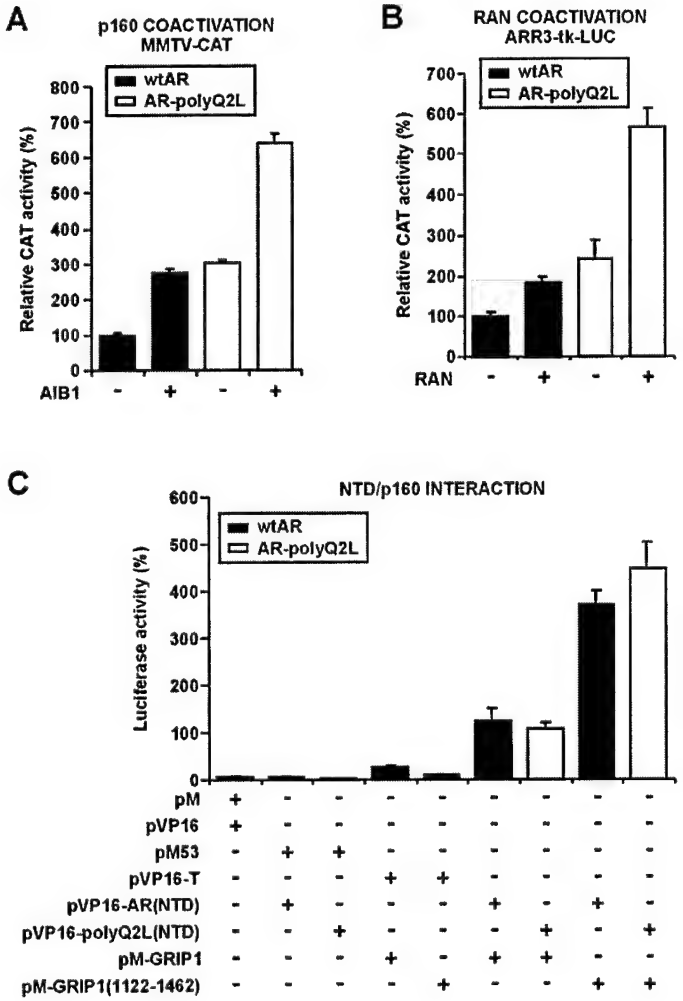


FIGURE 9

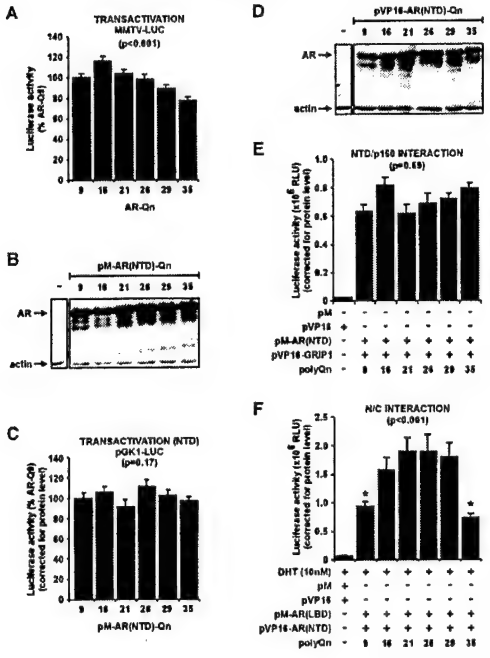


FIGURE 10

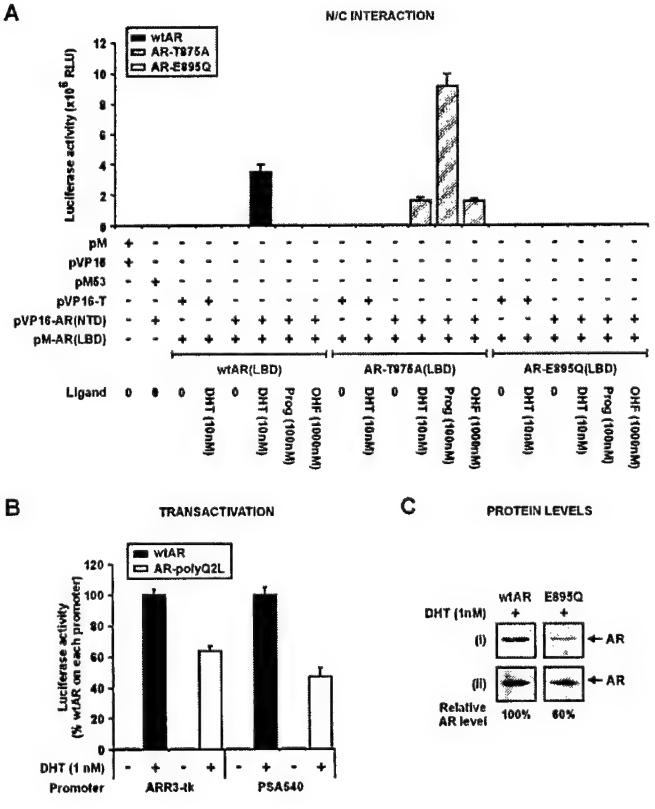
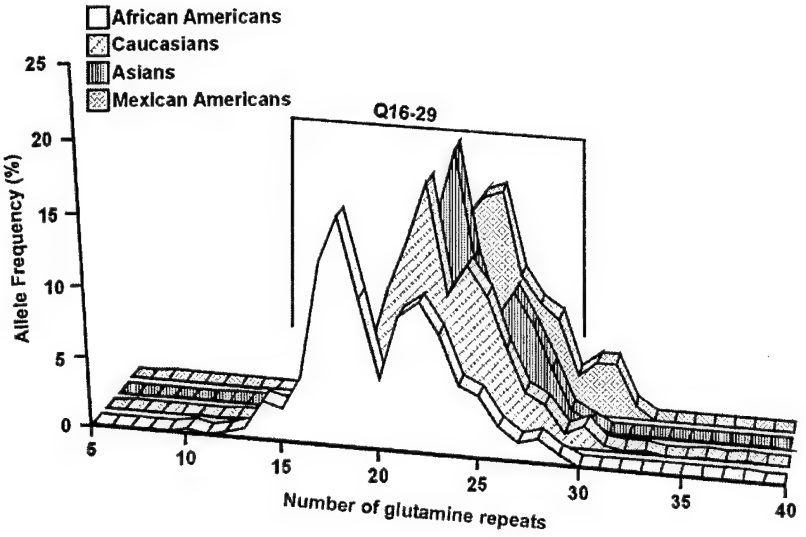


FIGURE 11

A



B

	African Americans	Caucasians	Asians	Mexican Americans
n	294	1111	136	205
Median Qn	19	22	23	25
Range Qn	10-29	10-43	16-30	16-32
Mean Qn	20	23	20	25
Qn<16	19 (6.5%)	8 (0.7%)	0 (0%)	0 (0%)
Qn16-29	275 (93.5%)	1661 (96.6%)	135 (99.3%)	187 (91.2%)
Qn>29	0 (0%)	30 (2.7%)	1 (0.7%)	18 (8.8%)
References	Edwards (14) Irvine (68)	Edwards (14) Irvine (68) Stanford (24) Spurdle (67)	Edwards (14) Irvine (68)	Edwards (14)

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Contribution of the androgen receptor to prostate cancer predisposition and progression

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Key words: androgen-signaling axis, CAG, GGC, mutation, androgen-ablation therapy

Abstract

Although prostate cancer is heterogeneous in its etiology and progression, androgen signaling through the androgen receptor (AR) appears to be involved in all aspects of the disease, from initiation to development of treatment resistance. Lifetime exposure to a constitutively more active AR, encoded by AR alleles as defined by two translated polymorphic microsatellites (CAG and GGC), results in a significant increase in prostate cancer risk. The AR gene is amplified or a target for somatic gain-of-function mutations in metastatic prostate cancer. Gain-of-function AR gene mutations may result in inappropriate activation of the AR, thereby contributing to the failure of conventional androgen-ablation treatments. In cases where no genetically altered receptors are observed, altered signaling through the AR, achieved by cross-talk with other signaling pathways (e.g. kinase-mediated pathways) and/or inappropriate expression of coregulatory proteins, may contribute to disease progression. Thus, the AR-signaling axis contributes to many aspects of prostate cancer, including initiation, progression and resistance to current forms of therapy. This recognition represents a paradigm shift in our understanding of the molecular mechanisms involved in progression of prostate cancer, and provides insight into novel AR-targeted therapies which ultimately may be more effective than current forms of androgen ablation.

Introduction

The development and maintenance of the normal prostate gland requires a functional androgen-signaling axis [1,2]. The primary components of this axis include testicular biosynthesis and transport of testosterone to target tissues, conversion of testosterone to its more active metabolite 5α-dihydrotestosterone (DHT), maturation of the androgen receptor (AR) to its ligand-binding competent form, and the subsequent transcriptional regulation of AR target genes. Through the AR, the androgen-signaling axis mediates diverse cellular functions in the prostate including differentiation, morphogenesis, angiogenesis, proliferation and apoptosis [1–5].

Prostate tumorigenesis also requires a functional androgen-signaling axis, the components of which form the principal targets of androgen-ablation therapies that inhibit the growth of prostate cancer. For patients who are either diagnosed with or subsequently develop metastatic disease, the only treatment option is androgen ablation (i.e., orchidectomy, treatment with LHRH agonists/antagonists and/or AR antagonists [6,7]). Despite an initial good response in 80–90% of patients with metastatic disease, androgen ablation is essentially palliative and disease progression eventually ensues [7,8]. Resistance to androgen ablation is not necessarily due to loss of androgen sensitivity, but may develop as a consequence of a deregulated androgen-signaling axis resulting from amplification or mutation of the AR gene, or ligand-independent activation (LIA) of the AR by growth factors and cytokines ([9–11]; reviewed in [12–14]).

Recent evidence suggests that the AR is involved in many phases of prostate cancer biology, including genetic predisposition (due to the existence of polymorphic variants), disease progression, and the development of resistance to androgen-ablation therapies. In this review, we document the contribution of the AR to each of these phases of prostate cancer.

Androgen receptor structure and function

The AR gene is located on the long arm of the X chromosome at Xq11–12, and comprises 8 exons that encode a protein of approximately 110 kDa (Figure 1). The AR can be broadly defined in terms of three distinct functional domains: a large amino-terminal transactivation domain (NTD) containing at least two strong constitutive transactivation functions; a DNA-binding domain (DBD); and a carboxy-terminal ligand-binding domain (LBD) that contains a highly conserved ligand-dependent transactivation function (AF-2) (Figure 1; [15]). The large NTD is encoded in its entirety by exon 1 of the gene and contains two polymorphic trinucleotide microsatellites, CAG and GGC, which encode variable-length polyglutamine (poly-Q) and polyglycine (poly-G) tracts, respectively, in the receptor (Figure 1). The CAG and GGC microsatellites have a normal size distribution of 6–39 and 7–20 repeats respectively [16,17]. The CAG and GGC microsatellites have expanded during primate evolution [18,19]. The Old World marmoset, drill and macaque monkeys, for example, possess only 3, 8 and 7 uninterrupted AR-CAG repeats, respectively [18], and the macaque and the prosimian lemur possess only 6 and 2 uninterrupted AR-GGC repeats, respectively [19]. Rubinsztein et al. [20] have shown that human microsatellite repeats statistically are more likely to be longer than their primate counterparts, suggesting that phylogenetic microsatellite expansion may be reflective of a mutational bias in favor of longer repeat lengths specifically in humans. A directional expansion of coding microsatellite repeats could be tolerated evolutionarily until it significantly alters function of the receptor such that reproduction is compromised.

Expansion of the CAG microsatellite to 40 or more repeats causes a rare, X-linked, adult onset, neurodegenerative disorder called spinal and bulbar

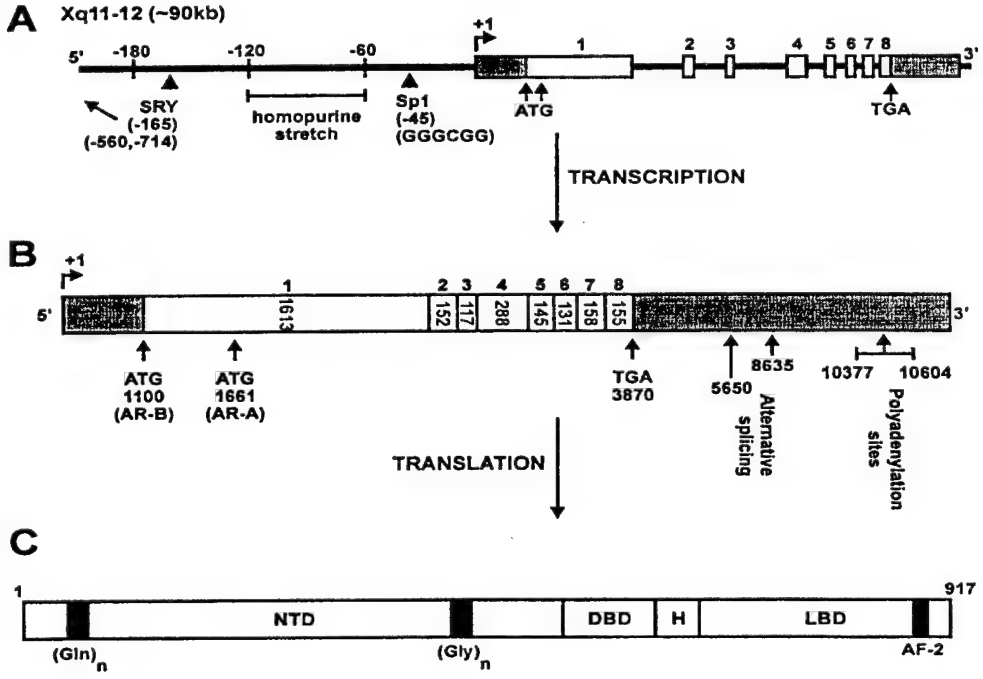

Figure 1. Transcription and translation of the AR. (A) Schematic representation of the AR gene structure on chromosome Xq11–12 showing important binding sites for SRY and SP1 transcription factors. Individual exons are separated by up to 16 kb of intronic sequence. (B) AR mRNA transcript showing alternative splice and polyadenylation sites. Translation is primarily directed from the first of two initiating methionine residues. (C) Structure of the predominant (AR-B) form of the AR. Indicated are the NTD, the DBD, the hinge region (H) and LBD. The positions of the three polymeric amino acid stretches in the NTD and the activation function, AF-2 in the LBD are shown (shaded).

muscular atrophy (SBMA) or Kennedy's disease [21,22]. In addition to progressive muscle weakness and atrophy due to loss of brain stem and spinal cord motor neurons, men with this disorder frequently present with symptoms of partial androgen insensitivity (i.e., gynecomastia and testicular atrophy), indicative of aberrant AR function [23,24]. Receptor proteins encoded by SBMA AR alleles have normal androgen-binding affinities but reduced transactivation capacity compared to wild-type AR [25,26]. Indeed, an inverse relationship between AR transactivation activity and CAG repeat length has been well-established over a CAG size range encompassing normal AR alleles [27–30].

Following translation of the AR, conformational maturation of steroid receptors by a multi-protein chaperone heterocomplex is essential for the acquisition of ligand-binding competence [31]. The specific details of this process for the AR are poorly defined, but in general, it requires at least three heat shock proteins, Hsp40, Hsp70 and Hsp90, and the co-chaperones p23 and Hop [31]. In the final stages of receptor maturation, Hsp90 becomes directly associated with the receptor LBD in a process stabilized by p23, and with one of the tetratricopeptide repeat (TPR) containing proteins which include the immunophilins FKBP51/52 and CyP40, and the protein–serine phosphatase, PP5 [31]. The interaction of the co-chaperone p23 with Hsp90 is an absolute requirement for heterocomplex stabilization of unliganded nuclear receptors [31]. Although many TPR containing proteins appear to have a similar affinity for Hsp90, they exhibit specific preferences for different steroid receptors, and may play a role in hormone action by altering the affinity and specificity for ligand. It is thought that the Hsp90-containing heterocomplex dynamically associates with steroid receptors to maintain them in a conformation that, although unstable, has a high affinity for ligand binding [31]. Following hormone binding in the cytoplasm, the Hsp90-containing heterocomplex is dissociated and the steroid receptor is rapidly translocated into the nucleus.

In the nucleus, the AR dimerizes and binds in the major groove of the DNA double-helix at specific DNA sequences called androgen response elements (AREs). The DNA bound AR dimer recruits a multi-protein complex containing members of the basal transcription machinery (e.g. TFIIF) and additional essential proteins termed cofactors, which act to up-regulate (coactivators) or inhibit (corepressors) target gene expression [32]. Chromatin remodeling occurs via targeted histone acetylation by recruited coactivators, resulting in the stable assembly of the pre-initiation transcriptional complex and enhanced rates of transcription initiation by RNA polymerase II [32]. Following ligand dissociation, the AR is shuttled back to the cytoplasm where it can re-associate with Hsp90 and ligand, subsequently undergoing multiple rounds of nucleocytoplasmic recycling and gene activation [33].

The DBD of steroid receptors contains two zinc finger motifs and a short C-terminal extension that form part of the hinge region [34,35]. Conserved amino acids in the second zinc finger of the DBD ('D' box) form a dimerization interface between steroid receptor monomers. The specificity of steroid receptors is determined at the level of DNA binding by conserved amino acids in the α-helix of the first zinc finger ('P' box) which contact specific base pairs in steroid receptor response elements [34]. Despite their diverse biological effects, the 'P' boxes of class I steroid receptors (i.e., AR and the corresponding receptors for glucocorticoids (GR), mineralocorticoids and progestins) are almost identical and the core DBD, which includes both zinc fingers, shares up to 73% identity [35]. However, sequence differences in the C-terminal extensions, which diverge considerably between steroid receptors (approximately 30% identity), may confer receptor selectivity for particular target sequences by allowing alternative modes of DNA binding [36]. Response elements for steroid receptors generally consist of hexameric half-sites arranged as either inverted repeats (symmetrically arranged palindromes) or direct repeats separated by three nucleotides. A recent analysis of AR-regulated genes has shown that the AR-responsive half-sites arranged as inverted repeats may induce head-to-head dimerization of the receptor, while the polarity of direct repeats may lead to head-to-tail dimerization [37–39]. These two distinct classes of AREs mediate cooperativity of AR binding and the unique regulation of target genes [40].

The contribution of AR-CAG size variation to prostate cancer risk

In 1992, Edwards et al. [16] reported the allelic frequency distribution of AR-CAG repeat size in different US racial–ethnic populations as part of a larger survey of genetic variation in a series of different trimeric and tetrameric tandem repeats. Among African-Americans, the frequency of AR alleles with

less than 22 CAG repeats was 65%, as compared to 53% in Caucasians and 34% in Asian-Americans. On the basis of these observations, we hypothesized that AR-CAG repeat length might be associated with the higher risk of prostate cancer in African-Americans, and the intermediate and low risk in Caucasions and Asian-Americans respectively, and that enhanced transcriptional activity of receptors with a shorter AR-CAG allele could promote tumorigenesis by enhancing prostatic epithelial cell turnover [41].

In 1995 we directly tested this hypothesis in a pilot case-control study comprising 68 prostate cancer patients and 123 control subjects [42]. In agreement with Edwards et al. [16] there was a prevalence of short AR-CAG alleles in African-American vs. Caucasian and Asian controls. In addition, modest though not statistically significant enrichment of short AR-CAG alleles was observed in the Caucasian prostate cancer patients. These findings were extended in an expanded follow-up study that showed a significantly higher prevalence of short AR-CAG alleles among prostate cancer patients, especially among those with advanced disease (Table 1; [43]). In addition to our studies, Hakimi et al. [44] identified a subgroup of patients diagnosed with advanced prostate cancer who had shorter AR-CAG repeats. Hardy et al. [45] furthermore, demonstrated an association between age of onset and AR-CAG repeat length.

Subsequently, several well-designed matched case-control studies demonstrated an approximate 2-fold increased prostate cancer risk, decreased age of onset and/or increased risk of advanced disease for reduced AR-CAG repeat length (Table 1). Giovannucci et al. [17] used a population selected from the Physicians Health Study that included 587 prostate cancer cases and 588 matched controls. The large sample size of this study allowed the authors to stratify cases by tumor grade and stage. A highly significant inverse correlation between AR-CAG repeat length and risk of developing prostate cancer was observed when repeat size was analyzed as a semi-continuous variable. Short AR-CAG alleles also correlated with an increased risk of having

Table 1. Studies evaluating the roles of the AR CAG and/or GGC microsatellites in prostate cancer risk, progression, and age at onset.

Study	Subjects	AR CAG repeat correlation with PCa			AR GGC repeat correlation with PCa		
		Risk	Stage/grade	Age at onset	Risk	Stage/grade	Age at onset
Pilot studies							
Irvine et al., 1995 [42]	US Caucasian	Yes	N/A	N/A	Yes	N/A	N/A
Hardy et al., 1996 [45]	US Caucasian	N/A	No	Yes	N/A	N/A	N/A
Ingles et al., 1997 [43]	US Caucasian	Yes	Yes	N/A	N/A	N/A	N/A
Hakimi et al., 1997 [44]	US Caucasian	Yes	Yes	No	Yes	No	No
Matched case-control studies							
Giovannucci et al., 1997 [17]	US Caucasian	Yes	Yes	No	N/A	N/A	N/A
Standford et al., 1997 [46]	US Caucasian	Yes	No	Yes	Yes	No	Yes
Platz et al., 1998 [53]	US Caucasian	N/A	N/A	N/A	Yes	N/A	N/A
Hsing et al., 2000 [47]	Chinese	Yes	No	No	Yes	No	No
Beilin et al., 2001 [30]	Australian White	No	No	Yes	N/A	N/A	N/A
Other studies							
Ekman et al., 1999 [49]	Swedish White	Yes	N/A	N/A	N/A	N/A	N/A
Edwards et al., 1999 [50]	British Caucasian	No	No	N/A	Yes	No	N/A
Correa-Cerro et al., 1999 [51]	French/German White	No	No	No	No	No	No
Bratt et al., 1999 [52]	Swedish White	No	Yes	Yes	N/A	N/A	N/A
Lange et al., 2000 [53]	US Caucasian (high risk)	No	No	No	N/A	N/A	N/A
Nam et al., 2000 [54]	Canadian	N/A	Yes	N/A	N/A	N/A	N/A
Latil et al., 2001 [55]	French White	No	No	Yes	N/A	N/A	N/A
Modugno et al., 2001 [56]	US Caucasian	Yes	N/A	N/A	N/A	N/A	N/A
Miller et al., 2001 [57]	US Caucasian	No	N/A	N/A	No	N/A	N/A
Panz et al., 2001 [58]	S. Africans (Black & White)	Yes	Yes	N/A	N/A	N/A	N/A

N/A, not applicable or not assessed; Yes, association between polymorphism and listed parameter; No, no significant association detected between polymorphism and listed parameter.

advanced disease, defined as a high-stage or high-grade tumor at diagnosis [17]. In another study, Stanford et al. [46] analyzed AR-CAG repeat length and prostate cancer risk in 301 prostate cancer cases and 277 matched controls [46]. They noted only a small increase in the frequency of AR-CAG alleles with less than 22 repeats in cancer patients compared with controls. Nevertheless, when AR-CAG repeat size was examined as a continuous variable, an overall age-adjusted relative odds of developing prostate cancer of 0.97 was observed for each additional CAG. More recently, Hsing et al. [47] reported that AR-CAG alleles were significantly shorter in prostate cancer patients compared to controls among Shanghai Chinese. This study is important as it was the first to demonstrate this association in a population group other than Caucasian. In a recent case-control study in an Australian Caucasian population [48], no association was observed between AR-CAG repeat length and prostate cancer risk, but a significant effect on the age of onset was observed. In other studies (Table 1), associations between AR-CAG repeat length and prostate cancer risk were not consistently observed, possibly due to small sample sizes, population differences and/or failure to appropriately match cases and controls [49–58].

While the consistent finding of the epidemiologic studies discussed above has provided evidence for an association between AR-CAG repeat length and prostate cancer risk, those studies did not address the molecular mechanisms underlying changes in receptor activity with length variation of the poly-Q tract (encoded by the polymorphic CAG repeat). As stated above, *in vitro* transient cotransfection studies have shown that ARs with longer poly-Q repeats have normal ligand-binding affinities but lower transactivation activities [25–28,48]. Protein expression levels are unlikely to account for this effect since they have been found to be similar for ARs containing between 9 and 42 poly-Q repeats [29]. However, two studies have reported that AR constructs with longer repeat lengths (CAG-50–52) are unstable and undergo accelerated degradation, potentially in a ligand-dependent manner [29,59]. The poly-Q size effect in AR transactivation activity observed in most *in vitro* studies is thought to be mediated, at least in part, through altered functional interactions with cofactors. In transient cotransfection experiments, the p160 coactivators, GRIP1, AIB1 and SRC-1 exaggerate the relative difference in AR transactivation activity with altered poly-Q length [29]. As the p160 coactivators bind to regions of the AR NTD distinct from the poly-Q tract, the size effect may be mediated by steric hindrance of p160–receptor interactions when poly-Q length is increased [29]. The RAS related G-protein, Ran/ARA24, which binds to the AR NTD in the region of the poly-Q, is an AR cofactor that appears to enhance AR activity in a poly-Q size-dependent manner [60]. Given the well-described role for Ran in protein nuclear transport, it is possible that larger poly-Q tracts inhibit the efficiency of Ran-directed AR nuclear import [61]. Clearly, more studies are required to determine whether the effects of other cofactors that act in a cell-, promoter- and/or AR-specific manner can be directly influenced by poly-Q length, and to determine how variation in AR poly-Q length can influence prostate cancer cell growth.

The contribution of AR-GGC size variation to prostate cancer risk

Allelic distributions of the GGC microsatellite are significantly different among racial–ethnic groups [42], with the 16-repeat GGC allele being least prevalent amongst high-risk African-Americans (i.e., 20%) and most prevalent in low-risk Asians (i.e., 70%). This is suggestive of a protective role for this allele in prostate cancer risk. It is possible that the 16-repeat GGC allele encodes an AR containing a poly-G tract of 'optimal' length for normal receptor function in prostatic epithelial cells. While this is speculative, as it is not known whether variation in poly-G length modulates AR activity, a weak though non-significant paucity of the 16-repeat GGC allele was observed among white Caucasian prostate cancer patients compared to control subjects, suggesting that there is enrichment of putative risk alleles (i.e., non-16-repeat GGC alleles) among cases [42].

Because the AR gene is X-linked, with each male inheriting a single maternal copy, it is possible to define a putative AR prostate cancer risk allelotype of short CAG (i.e., <22 repeats) and non-16-repeat GGC. As expected, we observed that the distribution of this allelotype was significantly different among control subjects, with African-Americans and Asians having the highest and lowest prevalence, respectively. Among white Caucasian prostate cancer patients, the <22 CAG/non-16-repeat GGC haplotype conferred a 2-fold increase in risk of prostate cancer, although statistical significance was not reached [42]. Among prostate cancer patients, a nonrandom distribution of CAG and GGC alleles was observed; 66% of patients with a short CAG allele also had a non-16-repeat GGC

allele, while only 25% of patients with long CAG alleles had a non-16-repeat GGC allele. As the CAG and GGC microsatellites are in close proximity at the AR locus, it was not surprising to find evidence of linkage disequilibrium between the intragenic markers in patient samples. In contrast, there was no evidence of linkage disequilibirium between control samples when assessed either together or by ethnicity. This indicates that in normal men, either one or both of the microsatellites are hypermutable, resulting in a random distribution of CAG and GGC alleles at the AR locus. Indeed, when the rate of mutation at the CAG microsatellite was measured using single-cell assays of sperm, an exceptionally high rate of 1–4% was observed [62]. Collectively, this data suggests that a non-random subset of CAG and GGC AR alleles occur in men with prostate cancer.

In three matched case-control studies (Table 1), a positive association between AR-GGC repeat length variation and prostate cancer risk was found [47,63,64]. The failure to consistently demonstrate this association in other studies (Table 1), might be due to the lack of statistical power and/or failure to appropriately match cases with controls. A more detailed assessment of the effects of the AR-GGC repeat on prostate cancer risk awaits elucidation of the effects of alterations in poly-G tract length on AR function.

AR and prostate cancer progression: Localized disease

Although the maintenance of AR immunoreactivity has been demonstrated in the majority of prostate tumors in both localized and metastatic disease [65–73], only recently has the role of AR in progression of clinically localized prostate cancer been addressed [71,73]. Henshall et al. [73] reported that AR was expressed in more than 70% of the tumor cells in localized prostate cancer, but that there was a loss of AR immunoreactivity in the adjacent peritumoral stroma which was associated with earlier relapse after radical prostatectomy. Another study by Sweat et al. [71] found no association between AR expression and disease progression in a highly selected cohort of tumors with a Gleason score of 6–9. In a recent study, we found that the level of AR protein in tumor foci determined by video image analysis is a strong predictor of the risk of relapse following radical prostatectomy (unpublished data). While further studies are necessary to determine how AR influences disease progression in clinically localized prostate cancer, a number of mechanisms have been identified in prostatic tumors that potentially explain the increase in levels of AR immunostaining observed in tumor cells in our study. These mechanisms include amplification of the AR gene [74], changes in the methylation status of the AR promoter and hence transcription of the AR gene [75,76], altered stability of AR mRNA [77] and LIA [11,78]. Irrespective of the mechanism, increased AR levels likely result in altered expression profiles of androgen-regulated proteins, including angiogenic factors, cell adhesion molecules and cell cycle regulators (e.g. vascular endothelial growth factor, integrins and cyclin-dependent kinases and their inhibitors [79–81], which collectively contribute to disease progression.

AR related mechanisms contributing to the failure of androgen-ablation therapy in advanced prostate cancer

Recent studies in clinical prostate cancer have identified several mechanisms that potentially explain how prostate tumors progress following initiation of androgen ablation, including amplification or mutation of the AR gene, and LIA of the AR ([9,82,83]; reviewed in [12,84–86]). These studies suggest that resistance to conventional hormonal therapy is not due to a loss of androgen sensitivity but rather may be a consequence of a deregulated androgen-signaling axis ([87]; reviewed in [84,85]. Although initial studies using the Dunning animal model suggested that loss of AR gene expression could be a mechanism for failure of androgen ablation [88,89], subsequent immunohistochemical studies of clinical prostate cancer have demonstrated that the AR is expressed in essentially all metastatic tumors, including those that continue to grow following androgen ablation [90]. Moreover, amplification of the AR gene has been reported in 22% of prostate cancer metastases [82], and in 23–28% of primary tumors following androgen deprivation [74,91]. An average 2-fold increased level of both AR and PSA proteins has been reported in prostate tumor samples with AR gene amplification compared to samples where no AR amplification was found [9,83]. Increased AR levels may augment the sensitivity of the androgen-signaling axis, and has the potential to contribute to disease progression during the course of androgen ablation.

The first indication that AR gene mutations might contribute to the failure of androgen-ablation therapies

came from studies of the androgen-responsive human prostate cancer cell line, LNCaP. The AR in LNCaP cells contains a single amino acid substitution (Thr–Ala877) that facilitates inappropriate activation by glucocorticoids, progestins, adrenal androgens, estradiol and the anti-androgen hydroxyflutamide [92,93]. Subsequently, somatic missense mutations have been detected throughout the AR coding sequence at frequencies of up to 50% in advanced primary tumors and metastatic deposits (reviewed in [12,94]). These mutations consistently result in receptors that exhibit decreased specificity of ligand-binding and enhanced receptor activation by androgens and non-classical ligands compared to wild-type AR (wtAR; reviewed in [85,95]). More recently in collaboration with Dr Norman Greenberg at Baylor College of Medicine, Houston, TX, we reported the identification of AR gene mutations in the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model [96]. Analogous to the findings in clinical prostate cancer, AR gene mutations detected in TRAMP tumors also result in receptors that contribute to altered androgen signaling [96].

Structural and functional collocation of AR variants

We recently reported that nearly 80% of missense AR gene mutations identified in clinical prostate cancer cluster to discrete regions of the receptor that collectively span less than 15% of the coding sequence ([85]; Figure 2; Table 2).

Ligand-binding domain variants. In the LBD, mutations collocate to (i) the 'signature sequence', a conserved 20-amino-acid region of nuclear receptors involved in ligand recognition and specificity [97], (ii) AF-2, a binding site for the p160 cofactors, and (iii) a region at the boundary of the hinge and LBD containing a 4-amino-acid tetrapeptide (^{668}QPIF^{671}) that may define a protein–protein interaction surface. Many of the AR gene mutations identified in the LBD of the AR in the TRAMP model occur in the same three regions as mutations in clinical prostate cancer. For example, a Phe–Ile671 mutation identified in an intact TRAMP mouse collocated to the ^{668}QPIF^{671} tetrapeptide with mutations identified human prostate cancer [10]. AR gene mutations identified in both clinical prostate cancer and TRAMP tumors in this region exhibit a 2–4-fold greater transactivation activity in response to DHT, non-classical ligands and hydroxyflutamide compared to wtAR (Figure 3A; [10]), without altering ligand-binding kinetics, receptor levels or DNA-binding capacity. Homology modeling revealed that the ^{668}QPIF^{671} tetrapeptide residues form a potential protein–protein interaction surface that is markedly disrupted by the naturally occurring mutations, providing a mechanism that could explain the observed gain in transcriptional activity [10].

Another AR missense mutation identified in the TRAMP model, Phe–Ser697, is located adjacent to the signature sequence. The Ser697 AR variant exhibits markedly reduced transactivation responses to progesterone and 17β-estradiol, but enhanced response to R1881 compared to wtAR (Figure 3B; [96]). These results are consistent with the role of the signature sequence in ligand recognition and specificity, and with previous reports of mutations in this region in clinical disease (reviewed in [12,85]). Analysis of the Thr–Ala877 AR variant, identified in a significant proportion of clinical prostate tumors and in the human prostate cancer cell line, LNCaP, has confirmed that this mutation exhibits increased transactivation activity in response to progesterone, 17β-estradiol, adrenal androgens and hydroxyflutamide compared to wtAR (Figure 3C; [98]). The recently determined AR-LBD crystal structure [99,100] allowed us to use homology modeling to demonstrate that this mutation results in changes to the shape and volume of the ligand-binding pocket such that bulkier ligands like progesterone can be accommodated [96].

DNA-binding domain variants. Five somatic missense mutations have been identified in clinical prostate tumors that collocate to a 14-amino-acid region at the carboxyl-terminal end of the first zinc finger motif in the DBD of the AR [101,102]. The effect of each of these mutations is unknown, but none of the codons in which they occur have been reported to contain mutations that cause receptor inactivation in the clinical syndrome of androgen insensitivity. Mutations in the AR-DBD have been shown to selectively affect transactivation and transrepression functions of the AR on different promoters despite a reduced DNA-binding ability [103,104], and may represent a predisposing factor for male breast cancer [105]. Due to the high homology of the DBD across members of the nuclear receptor superfamily, the cell and promoter specificity of different receptors is, in part,

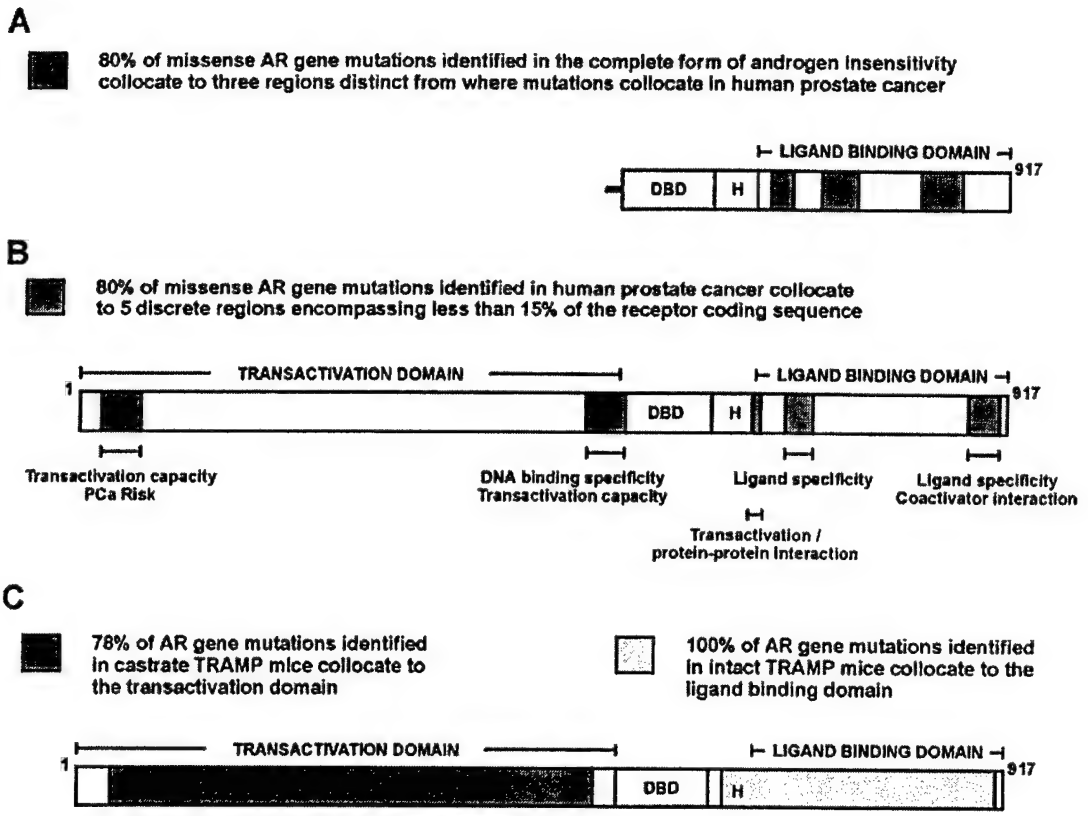

Figure 2. Collocation of AR variants. (A) Collocation of 80% of inactivating AR gene mutations detected in the clinical syndrome of complete androgen insensitivity. (B) Collocation of 80% of AR gene mutations detected in clinical prostate cancer to five discrete regions of the receptor (shaded), which account for less than 15% of the coding sequence. (C) AR gene mutations detected in prostate tumors derived from the TRAMP model segregate to the amino-terminal transactivation domain or to the LBD in castrated and intact mice respectively.

mediated by only a few changes in DBD sequence [106]. It has been speculated that mutations in the DBD could result in AR variants that bind to response elements normally specific for other nuclear receptors [105], leading to inappropriate activation or repression of growth regulatory pathways. In an analogous manner, mutations in androgen receptor response elements have been shown to increase the sensitivity of the enhancer for the glucocorticoid receptor [107]. Modeling experiments suggest that residues in the AR-DBD could form a protein interaction surface [105], and several AR coactivators that interact with the DBD in a ligand-dependent manner [108–110] are predicted to alter receptor activity via local chromatin remodeling [108], interaction with components of the transcriptional machinery [109], or inhibiting nuclear export [111]. It is also possible that mutations in the DBD of the AR gene identified in prostate cancer could alter the affinity of receptor binding to response elements, resulting in altered expression of a range of target genes regulated by the AR.

Amino-terminal transactivation domain variants. Nearly half of the AR gene mutations identified in clinical prostate cancer are located in the NTD of the receptor. AR gene mutations in this domain have also been identified in the TRAMP model and, analogous to the observations for the LBD, these mutations cluster with those identified in clinical prostate cancer to discrete regions within the NTD that are implicated in receptor function. The main regions of collocation in the NTD are (i) within and adjacent to the poly-Q tract

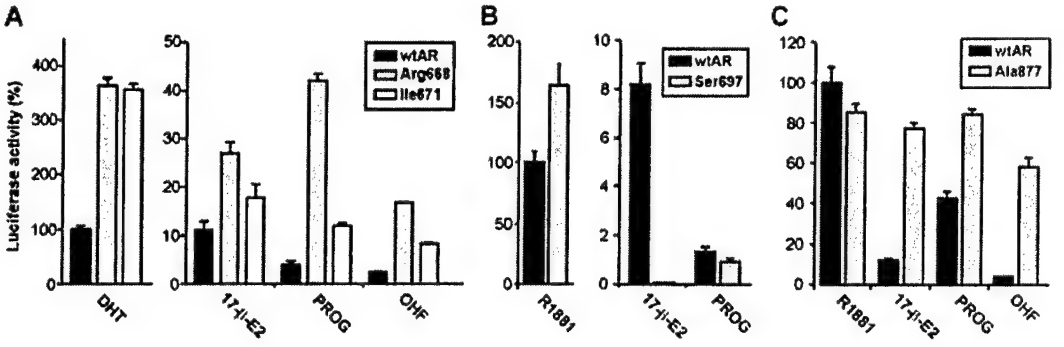

Figure 3. Transactivation capacity of AR variants identified in tumors derived from clinical prostate cancer (Arg668, Ala877) and TRAMP mice (Ile671, Ser697). Transactivation assays were performed in the human prostate cancer cell line, PC-3 with the minimal androgen-responsive probasin promoter, tk81-PB3 as previously described (10). Data is expressed as a percentage of the luciferase activity induced by wtAR in the presence of 1 nM DHT, and represents the mean (±sem) of 3–7 independent experiments. (A) Mutations occurring in ^{668}QPIF^{671} residues. Treatment was with 1 nM of either DHT, 17β-estradiol (17β-E2), or progesterone (PROG), or with 1 μM hydroxyflutamide (OHF). (B) Mutation occurring adjacent to the signature sequence. Treatment was with 1 nM R1881 or 10 nM of either 17β-E2 or PROG. (C) LNCaP AR variant in the region of AF-2. Treatment was with 1 nM of R1881 or 10 nM of either 17β-E2 or PROG, or with 1000 nM OHF.

(codons 54–78), which as discussed above has been implicated in modulating receptor activity and prostate cancer risk, and (ii) a region amino-terminal to the DBD (codons 502–535) known to modulate the transactivation capacity of the receptor in both ligand-dependent and ligand-independent manner (i.e., a LIA function or LIAF) [112,113] (Figure 2).

Somatic contractions in the CAG repeat of the AR gene, which potentially increase AR activity in a subpopulation of cells and thereby contribute to disease progression, have been identified in three independent studies of clinical prostate tumors [114–116]. In addition, we have recently identified a somatic mutation within the CAG repeat of the AR gene in a primary prostate tumor that results in interruption of the polyglutamine repeat by two leucine residues. This AR variant has a 2–4-fold greater ability to transactivate target genes compared to wtAR in the presence of physiological concentrations of DHT [117]. Four additional somatic mutations have been identified in or adjacent to the CAG repeat region of the AR gene in human prostate cancer [101], but have not yet been characterized. Further analysis of inherited and somatic alterations in this region of the AR gene in prostate cancer is warranted to determine the contribution of this motif to AR activity, and its potential to influence the development and/or progression of the disease.

A second region of the NTD where amino acid substitutions have been identified in hormone-refractory prostate tumors is the LIAF. In a recent study, we examined the complete coding sequence of the AR gene for mutations in metastatic tissue biopsies from 12 patients who exhibited the clinical syndrome of steroid-hormone and anti-antiandrogen withdrawal response to hydroxyflutamide. Four of seven mutations identified in the tumor samples collocated to a small carboxyl-terminal portion (codons 502–535) of the NTD of the AR. An additional mutation (Asp–Gly526) previously identified in our studies of primary prostate tumors [101], and another identified in the TRAMP model [96], collocate to this region of the AR. This carboxyl-terminal region of the amino-terminal domain of the AR is known to modulate the transactivation capacity of the receptor in both a ligand-dependent and ligand-independent manner [112,113], and has recently been shown to be involved in direct interactions with the p160 coactivators and the transcription regulator p300/CBP [29,118,119]. This region of the NTD also contains the binding site for the receptor accessory factor (RAF), which enhances the specific DNA binding of rat AR [120]. Further evidence in support of the LIAF region being important in AR transactivation has been provided by recent studies demonstrating that mutation of Ser513, which is located in a consensus MAP kinase phosphorylation site, partially inhibits LIA of the receptor by HER-2/*neu*. Collectively, these observations suggest that the LIAF region may contain an interaction surface for accessory proteins that

promote ligand-independent transactivation, and that mutations in this region may alter the ability of the receptor to respond to these and other cofactors, thereby altering the transactivation capacity of the AR in a manner that could provide a growth advantage to prostate cancer cells in an appropriate hormonal environment.

Contribution of cofactors to AR signaling

Two additional AR NTD missense mutations (Met–Thr265; Pro–Ser268; [101]) identified in clinical prostate cancer are located in close proximity to two mutations (Ala–Thr234; Glu–Gly236; [96]) identified in TRAMP tumors. Characterization of the Glu–Gly236 substitution revealed that the variant receptor had increased transactivation function compared to wtAR in response to R1881 and 17β-estradiol only in the presence of the coactivator, ARA70, and increased response to R1881 but not 17β-estradiol in the presence of the coactivator, ARA160 [96]. Similarly, Gregory et al. [121] have shown that overexpression of the p160 coactivators, TIF2/GRIP1 and SRC-1, observed in recurrent tumors from CWR22 human prostate xenografts and clinical prostate cancer, increases AR transactivation capacity at physiological concentrations of non-classical ligands (adrenal androgens, estradiol and progesterone). Similarly, Ye *et al.* [143] have shown that coactivators (e.g. ARA70, ARA160) can enhance the androgenic activity of 17β-estradiol and hydroxyflutamide, suggesting that the effect of agonists and antiandrogens can be modulated by accessory proteins. Collectively, these findings suggest that the phenotype of some AR gene mutations may only be apparent in the presence of the appropriate milieu of coregulators, and that altered expression and/or structure of AR accessory proteins in prostate cancer cells could provide another mechanism contributing to the recurrent growth of prostate tumors in an androgen-depleted environment.

Ligand-independent activation of the AR

Another potentially important mechanism contributing to the failure of androgen ablation is LIA of the AR. The AR can be activated in the absence of ligand by growth factors (keratinocyte growth factor, insulin-like growth factor-1 and epidermal growth factor), cytokines (Interleukin-6), protein kinase-A, components of the MAP kinase pathway (MEKK1), differentiation agents such as butyrate and other factors that directly or indirectly increase intracellular kinase activity or decrease phosphatase activity (reviewed in [84]).

Aberrant expression of growth factor receptors also contributes to development and progression of prostate cancers [12,85]. HER2 (neu/c-erbB-2), a transmembrane glycoprotein member of the epidermal growth factor receptor family, is overexpressed in carcinomas of the breast, ovary, and stomach [17,42,122]. Unlike other epidermal growth factor receptor members, HER2 has intrinsic tyrosine kinase activity and mediates signal transduction in the absence of ligand [123]. Recent studies suggest that HER2 expression is increased in hormone-refractory prostate tumors compared to earlier stages of disease [124,125]. Over-expression of HER2 in androgen-responsive prostate cancer cell lines enhances AR transactivation of androgen-regulated genes such as PSA, in a ligand-independent manner, and increases cell survival during androgen deprivation [11,126]. Although the mechanism involved in HER2 modulation of AR transactivation has not been fully characterized, HER2 expression is associated with activation of MAP kinase and Akt (protein kinase B) pathways which have been implicated in LIA of the AR [11,78]. It was recently shown that the tyrosine kinase receptor, HER-2/*neu,* can promote LIA of the AR via both the PI3K/Akt and MAP kinase pathways [78,127]. In those studies, LIA by HER-2/*neu* could be partially blocked by an inhibitor of the PI3K Akt pathway [78], or mutation of AR-Ser513, which is located in a consensus MAP kinase phosphorylation site. HER-2/*neu* activation of Akt results in binding of Akt to the AR and phosphorylation of the receptor at two residues (Ser212, Ser791). Another study demonstrated that a specific inhibitor of protein kinase A could block LIA of the AR induced by butyrate [127]. These data suggest that both ligand-dependent and ligand-independent signals converge upon the AR, with at least three signal transduction pathways having the potential to activate the AR.

Tumor cells with increased HER2 expression and high AR may have a selective growth advantage. For example HER2 activation of an Akt–AR pathway [11,78,126] may confer a clonal advantage by promoting cancer cell survival via the androgen-signaling axis [78] or by the induction of Akt-dependent pathways [123]. In a recent study using prostate cancer xenograft models, Herceptin (a monoclonal antibody directed against activated HER2) monotherapy resulted in anti-proliferative activity in androgen-dependent LNCaP and CWR22 tumors, but no significant growth

inhibition was observed in androgen-independent CWR22 tumors [128]. The lack of response of the androgen-independent tumors to Herceptin in the presence of androgen indicates that signaling through the AR is a requisite for Herceptin response in these prostate tumors [128]. Thus, patients with elevated levels of both HER2 and AR immunostaining may benefit from early treatment targeting both the AR and HER2 signaling cascades.

Therapy selects for AR gene mutations with a phenotype permissive for growth

In the late 1980s, Labrie et al. [129] reported that administration of LHRH agonists in combination with hydroxyflutamide (androgen blockade; CAB) could prolong survival of patients with metastatic prostate cancer by about 17 months. However, subsequent reports were conflicting, and currently most patients initially are treated with monotherapy, usually LHRH agonists. Indeed, a recent systematic review of CAB, encompassing 20 individual trials and more than 6000 patients, concluded that CAB results in only a modest increase in survival compared to monotherapy alone, but is more likely to be associated with adverse events and reduced quality of life [130]. Recent evidence regarding the mechanisms contributing to therapy failure (see above) suggests that combinational approaches with LHRH agonists and receptor antagonists cannot completely abrogate androgen action, and may select for cells with a growth state permissive for a particular hormonal environment. The potential clinical importance of a therapy-mediated selective pressure is illustrated by the syndrome of steroid-hormone and anti-antiandrogen withdrawal, which is characterized by tumor regression and decreasing serum levels of PSA when treatment with an anti-androgen, progestational agent or estrogen is selectively discontinued at a time of clinical progression [131]. A withdrawal response has been observed in up to 30% of patients with hormone-refractory prostate cancer when treatment with the anti-androgen, hydroxyflutamide is terminated [131–133], and has also been documented following withdrawal of the AR antagonists, nilutamide and bicalutamide, the estrogens, diethylstilbestrol and megestrol acetate, and the progestational agent, chlormadinone acetate [134–139]. Withdrawal responses have been reported at a higher incidence following combined therapy, consisting of castration or LHRH agonists in combination with an AR antagonist, compared to antagonist alone, leading the authors to conclude that prolonged exposure to antiandrogens was the predominant factor in the withdrawal response rather than a low level of androgens [135,140]. In one study, inhibition of adrenal steroid production with ketoconazole following discontinuation of antiandrogen therapy resulted in a higher proportion of patients (55%) exhibiting a withdrawal response and an increased duration of response [141] than reported for withdrawal of the antiandrogen alone [140].

Selection for AR gene mutations with a phenotype permissive for growth is also evident from a number of other studies. AR gene mutations detected in patients who were treated with hydroxyflutamide in conjunction with androgen-ablation therapy result in receptors exhibiting a marked increase in activity in response to hydroxyflutamide, but not to DHT or other androgenic ligands [142]. In the TRAMP model, we recently reported that different hormonal environments result in the selection of AR variants with mutations in distinctly different regions of the receptor [96]. In tumors derived from TRAMP mice at 24–28 weeks of age, 7/7 of the missense AR gene mutations identified in the amino-terminal transactivation domain were derived from mice castrated at 12 weeks of age, whereas 6/8 of the mutations identified in the LBD were from intact animals ([96]; Figure 2C). Moreover, 4/9 of the mutations identified in castrated TRAMP mice resulted in receptors with increased transactivation function in the absence of ligand [96]. Therefore, AR gene mutations identified in prostate cancer could provide a selective growth advantage given the appropriate hormonal environment, resulting in the re-emergence of tumor growth during the course of hormone-ablation therapies. In addition, AR gene amplification and overexpression of the AR has been reported in hormone-refractory prostate cancer following monotherapy but not in primary prostate tumors [9], suggesting an alternative mechanism by which hormonal therapies could select for cells with an ability to maintain growth during treatment.

Conclusions

Collectively, the above evidence suggests that AR signaling plays a key role in many phases of prostate cancer biology. Carefully matched case-control and other studies suggest that length variation of the AR-CAG and AR-GGC polymorphic microsatellite repeats contributes to prostate cancer risk, and may

also influence age of onset and tumor pathology, by altering AR transcriptional activity and/or interaction with AR coregulators. AR gene mutations have frequently been reported in clinical prostate cancer and consistently exhibit a gain-of-function phenotype that, along with AR gene amplification and/or activation of the AR by growth factors and cytokines, could facilitate continued AR signaling in an androgen-depleted environment. Thus, resistance to androgen ablation and survival of prostate cancer cells is not necessarily due to the evolution of a growth state that circumvents the androgen-signaling axis, but could be explained in part by increased activity of the AR in the presence of native ligands, inappropriate activation of the AR by non-classical ligands due to mutations in the AR gene or inappropriate expression of AR coregulators, or ligand-independent mechanisms. This represents a paradigm shift in our understanding of hormone-refractory prostate cancer. Further analysis of AR target genes and how their transcription is influenced by non-classical ligands, AR coregulators or variation of the polymorphic AR-CAG and AR-GGC repeats is necessary in order to develop new treatment strategies that target androgen signaling, irrespective of the structure and level of expression of the receptor. This potentially could result in a more complete blockade of androgen signaling, which would represent a significant advance in the treatment of metastatic prostate cancer by preventing or delaying the onset of resistance to androgen-ablation therapies.

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EDITORIAL

Size of the Androgen Receptor CAG Repeat and Prostate Cancer: Does It Matter?

THE PRECISE genetic contribution to prostate cancer predisposition and progression remains an elusive target.[1] Although several genetic loci have been identified, the heterogeneity of the disease within and among geographically or racially defined family cohorts has precluded the identification of a single high-penetrance locus or gene. Within a polygenetic model for prostate cancer,[2] the androgen receptor (*AR*) is a strong candidate gene influencing the onset and progression of the disease. The report by Bennett et al[3] in this issue of the *Journal of Clinical Oncology* addresses the important topic of CAG size variation in the *AR* gene in relation to the clinical outcome of prostate cancer among African-Americans and whites.

Men inherit one allele of the *AR* gene with the maternal X chromosome. Size variation of a polymorphic CAG microsatellite in exon 1 of the gene modifies prostate cancer risk and progression.[4] This genotype-phenotype association was first hypothesized on the basis of four considerations[5,6] (Fig 1). The allelic frequency distribution of *AR*-CAG repeat size in different racial or ethnic populations in the United States relates to prostate cancer risk. Compared with white and Asian-American men, African-American men have, on average, shorter *AR*-CAG alleles and higher prostate cancer risk. Men with abnormally large *AR*-CAG sizes have spinobulbar muscular atrophy with associated partial androgen insensitivity, establishing an inverse link between *AR*-CAG repeat size and *AR* activity. In addition, *AR*s with shorter CAG repeats have higher transactivation activities in vitro. Finally, long-term exposure of prostate cells to increased *AR* activity might cause increased proliferation and lead to oncogenic transformation. Indeed, over the last several years, numerous epidemiologic—albeit not all—and laboratory-based studies have supported a causal link between the *AR*-CAG size and prostate cancer predisposition and/or progression.[4] Most of these studies, however, have focused on white populations. In this issue, Bennett et al compared African-American and white veterans from the Veterans Affairs Cancer of the Prostate Outcomes Study with respect to *AR*-CAG size, prostate-specific antigen (PSA) level, Gleason score, and cancer stage. Their main findings are that short *AR*-CAG repeat length is more frequent in African-American men and associated with the development of metastatic disease but not with other clinical or pathologic end points. Among their patients, African-American men presented with more advanced disease, as evidenced by a higher percentage with stage D disease (53.5 *v* 31.3%) and higher PSA levels (29.2 *v* 10.3 ng/mL), compared with white men.

In genetic association studies, it is important to recognize that polymorphisms with minor alleles occurring at relatively high frequency in the general population likely have subtle phenotypic effects due to evolutionary pressures. Genetic alterations with strong phenotypic effects often are selected for, or against, over time, which results in low frequencies of certain alleles in the general population. This is particularly true for the *AR*, which has direct reproductive fitness implications, since its activity relates directly to male sexual reproduction. In most well-controlled observational studies to date, a modest but statistically significant association between *AR*-CAG repeat length and prostate cancer risk has been observed.[4] In other studies, an association between *AR*-CAG repeat length and prostate cancer risk has not been observed, perhaps in some cases because of small sample sizes and in others because of poor strategies for selecting a control comparison group.

Despite these uncertainties, the *AR* remains a prime candidate for influencing not only prostate cancer predisposition but also its progression. The *AR* plays a role during all stages of prostate cancer development, even in so-called androgen-independent disease. A consistent finding is that expression of the *AR* is maintained in virtually all advanced prostate tumors. Increased expression or gain of function mutations in the *AR* gene occurs frequently. These findings have provided compelling evidence that ultimate failure of androgen ablation therapy for metastatic prostate cancer does not result from loss of androgen signaling but rather from the acquisition of changes that lead to the activation of the androgen-signaling axis in the absence of ligand. Other mechanisms potentially contributing to alterations in *AR* signaling in prostate cancer include inappropriate interaction with coregulatory proteins and ligand-independent activation of the *AR* by growth factors, receptor tyrosine kinases, and cytokines.

A second important consideration in genetic epidemiologic studies is that the phenotype, such as prostate cancer in the present context, is often complex and heterogeneous in nature. This results in imprecise groupings, even within defined clinical or pathologic strata or within racial or ethnic groups. Indeed, in certain racial or ethnic groups in the United States, there is considerable ethnic admixture, which might confound results; this form of confounding is known as population stratification.[7,8] For example, if an increased frequency of shorter *AR*-CAG repeat length is associated

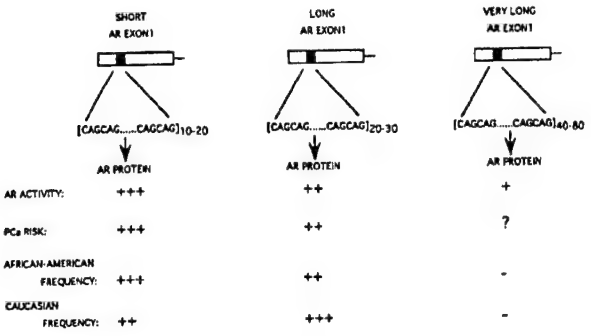

Fig 1. Genotype-phenotype association.

with Africans and if Africans have a higher risk of prostate cancer, then an association of shorter *AR*-CAG repeat length with prostate cancer might simply reflect the "Africanness" of the African-American population without having a direct cause–effect relationship. In the Bennett et al[3] article, a different form of population stratification occurred in the analysis. More African-Americans presented with stage D disease than white patients. African-American cases, furthermore, had on average shorter *AR*-CAG repeats than did the white cases. Therefore, it is not surprising that among African-Americans and whites combined, stage D disease was associated with shorter *AR*-CAG repeat length (although not statistically significant, $P = .09$) simply because more African-Americans presented with stage D disease in the first place. As expected, the logistic regression analysis revealed only African-American race and associated elevated PSA levels at diagnosis as significant independent predictors of advanced-stage disease.

Although much of the discrepancy among the many reports relating *AR*-CAG size with prostate cancer risk or progression is undoubtedly due to poor study design, the problem is exacerbated by a lack of precise molecular understanding of the effects of varying polyglutamine size (encoded by the CAG repeat) on *AR* function. Transient transfection studies in cultured cells have established that *AR* transactivation activity is inversely modulated by polyglutamine size, with longer repeats conferring lower activity.[4] However, a detailed analysis of the physical interactions and cross-modulation between interacting proteins during the *AR* signaling mechanism is still lacking. Such information undoubtedly will contribute to a better understanding of *AR* polymorphisms and prostate cancer risk associations.

The study by Bennett et al[3] has extended the racial–ethnic difference in *AR*-CAG size distribution between African-Americans and whites to prostate cancer cases. Furthermore, it has highlighted the distinct disease characteristics at presentation between African-American and white patients within the same Veterans Affairs health care system. Finally, Bennett et al have shown that among prostate cancer patients, *AR*-CAG size does matter, albeit simply in their case to mark the racial–ethnic origin of the patient.

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Androgen Receptor Signaling in Prostate Cancer

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Introduction

The androgen-signaling axis is the principal regulator of the development, function and growth of the prostate gland, and also plays a vital role in prostate cancer predisposition and progression. The major components of this axis includes the biosynthesis and transport of testosterone to target tissues where it is converted to the more active metabolite 5α-dihydrotestosterone (DHT), maturation of the androgen receptor (AR) to a ligand-binding competent form, and the subsequent transcriptional regulation of AR target genes. The AR, which is the pivotal component of androgen signaling, is a member of the superfamily of nuclear transcription factors that regulate a diverse range of cellular functions by providing a direct link between signaling molecules and gene transcription [1-3]. The AR is unique among nuclear receptors in that a strong constitutive transactivation function involving at least 3 overlapping regions of the N-terminal domain (NTD) is responsible for most, if not all, its transactivation activity. The AR-NTD also contains two polymorphic regions (polyQ and polyG stretches) that are associated with prostate cancer predisposition. In particular, there is an inverse correlation between polyQ size and both receptor activity and risk of developing prostate cancer.

The status of the androgen-signaling axis is especially important in patients who are either diagnosed with, or subsequently develop, metastatic prostate cancer. Currently, the success of the only treatment option for metastatic disease, androgen ablation (i.e. orchidectomy, treatment with LHRH agoniss/antagonists and/or AR antagonists [4, 5]), is dependent on a functional androgen signaling axis. Despite an initial response to androgen ablation in 80-90% of patients with metastatic disease, these hormonal therapies are essentially palliative and disease progression eventually ensues [5, 6]. Recent studies indicate that resistance to androgen ablation is not necessarily due to a loss of androgen sensitivity, but may develop as a consequence of a deregulated androgen signaling axis resulting from increased levels of, or gain of function mutations in, the AR gene, altered interaction between the receptor and coregulatory molecules, or ligand-independent activation of the AR by

growth factors and cytokines [7-9] (reviewed in [10-12]). In this chapter, we review the contribution of molecular aberrations in the AR gene to genetic predisposition, tumor progression and the development of resistance to androgen-ablation therapies. These new insights into AR signaling in prostate cancer provide a unifying conceptual framework for understanding the development of resistance to androgen-ablation therapies.

Androgen receptor structure and function

The AR is a member of the superfamily of nuclear transcription factors which consists of more than 300 members across vertebrates, arthropods and nematodes, and includes receptors for steroid hormones, vitamin D, retinoic acids and thyroid hormones, as well as a number of 'orphan' receptors for which no ligand has yet been identified [1]. Specifically, the AR is a member of Class III nuclear receptors, including those for estrogens, progestins, glucocorticoids, and mineralocorticoids, and the orphan estrogen-related receptors, which represent the terminal derivatives of cholesterol biosynthesis [3]. Like many other nuclear receptors (eg glucocorticoid, progesterone and estrogen receptors), the AR contains a ligand-dependent activation function, AF2 in helix 12 of the LBD that interacts predominantly with LxxLL motifs of the p160 coactivators. A key functional role for AF2 is to recruit, in a ligand-dependent manner, chromatin-remodeling factors that may also bridge or stabilize the interaction between the DNA bound AR and proteins in the basal transcription complex.

Gene and protein structure The human AR is encoded by a large single copy gene (>90kb), located on the long arm of the X chromosome at Xq11-12, that is organised into eight exons divided by long intronic sequences [13-15] (Figure 1). Although the AR promoter lacks classic TATA or CAAT box elements, there are more than 22 potential binding sites for known transcription factors, including Sp1, the cAMP response element binding protein factor, SRY and NFκB, and a critical homopurine/homopyrimidine stretch adjacent to the functional GC box that directs efficient recruitment of TFIID [16]. Although the AR gene appears to be ubiquitously expressed, the extent of expression in various tissues differs by 2-3 orders of magnitude [17].

Two AR mRNA species of approximately 10.5kb and 7.5kb, which arise from alternative splicing of the AR transcript, have been identified in human tissues, with the 10.5kb form observed at significantly greater abundance [18] (Figure 1). The AR coding sequence of approximately 3.0kb gives rise to two naturally occurring AR isoforms by translation initiation at two methionine residues [19]. The predominant isoform, AR-B, is an approximately 917 amino acid protein with a molecular weight of 98.9kDa [14 15, 20](Figure 1). The smaller AR-A isoform lacks the N-terminal 187 amino acids of AR-B and has a molecular weight of 80kDa. Although both isoforms coexist in a variety of adult tissues, AR-A is present at about 1/10th of the level of AR-B [19]. Post-translational modifications, including phosphorylation, account for the observed size of AR-A (~87kDa) and AR-B (~110kDa) on SDS polyacrylamide gel electrophoresis.

The AR protein can be defined broadly in terms of three major functional domains; a large amino-terminal domain (NTD) encoded entirely by exon 1; a DNA-binding domain (DBD) encoded by exons 2 and 3; and a carboxy-terminal ligand binding domain (LBD) encoded by exons 4-8 that contains a highly conserved ligand-dependent transactivation function (AF-2) (Figure 1) [21]. The DBD and LBD are highly conserved amongst the steroid receptors while the NTD and the hinge, which is a small region separating the DBD from the LBD encoded by the 5' portion of exon 4, show poor homology with the corresponding domains of other receptors. Amino terminal transactivation domain The large NTD of the AR is unique amongst nuclear receptors in that it is responsible for almost the entire transactivation potential of the protein. It contains at least three overlapping constitutive transactivation domains that, in the context of the full-length receptor, are activated or inhibited by conformational changes resulting from hormone binding at the LBD or by other mechanisms. In particular, two activation functions AF-1 (aa 51-210) and AF-5 (aa 369-492), which contain LxxLL-like motifs, appear to cooperate to facilitate ligand induced N- and C- interactions, the latter with an interaction surface that overlaps with AF-2 in helix 12 of the LBD (Figure 1). Access to the hydrophobic binding surface of LxxLL-like peptides of coactivators also is blocked by helix 12 of the LBD. An N/C-interaction can occur intra- or inter-molecularly, and is associated with increased affinity of receptor binding to androgen response elements of target gene promoters, and stabilization of the receptor complex. It is not clear if there is functional redundancy between AF1 and AF5 in the AR-NTD and AF2 in the LBD, or whether both NTD activation functions are absolutely required for recruitment of coregulatory factors and transcription initiation. Recently, we and others have shown that p160 cofactors can form a bridge to tether the AR N-C termini via an interaction with a glutamine rich region of the cofactor and the LxxLL-like motifs in the AR-NTD, and the LxxLL motifs of the cofactor and AF2 in the AR [22, 23]. It is proposed that this interaction is promoted by the cofactor to stabilize the receptor for maximal activity.

The transactivation potential of subdomains in the AR-NTD is modulated by two polymorphic trinucleotide microsatellite repeats, CAG and GGN in the coding sequence. The normal size distribution of these microsatellites in the population is 9-39 CAG and 14-27 GGN repeats, with a modal repeat size of 21 and 23,

respectively [24, 25]. These repeats encode variable-length polyglutamine (poly-Q) and polyglycine (poly-G) tracts, respectively, in the receptor (Figure 1). The NTD also contains a strong ligand-independent activation function (LIAF, aa 502-535; see below) located downstream of AF-5 (Figure 1) which, on the basis of recent studies, appears to be silenced by interaction with an inhibitory subdomain (ISD) in the NTD containing the poly-Q region [22].

Expansion of the CAG microsatellite to 40 or more repeats causes a rare, X-linked, adult onset, neurodegenerative disorder called spinal and bulbar muscular atrophy (SBMA) or Kennedy's disease [26, 27]. In addition to progressive muscle weakness and atrophy due to loss of brain stem and spinal cord motor neurons, men with this disorder frequently present with symptoms of partial androgen insensitivity (i.e., gynecomastia and testicular atrophy), indicative of aberrant AR function [28, 29]. Receptor proteins encoded by SBMA AR alleles have normal androgen-binding affinities but reduced transactivation capacity compared to wild type AR [30, 31]. Indeed, an inverse relationship between AR transactivation activity and CAG repeat length has been well established over a CAG size range encompassing normal AR alleles [22, 32-34].

Ligand binding domain The ability of steroid receptors to respond rapidly to hormone signaling events in a highly sensitive and specific manner resides in their ability to discriminate between low circulating levels of structurally similar hormones. Remarkably, this facility is bestowed by the highly homologous LBDs of these receptors. The recently resolved crystal structure determined that the AR-LBD, like those of other steroid receptors, consists of 11 alpha helices (as with PR, the AR LBD has no helix 2) and four short beta sheets arranged in a 'helical-sandwich' around the protected, relatively compact ligand binding pocket (LBP; Figure 2) [35-37]. High affinity ligands such as DHT, testosterone and the synthetic androgen, R1881, make direct contact with 18 individual amino acids in helices 3, 4, 5, 7 and 11, and with one of the beta sheets. Although the crystal structure of the apo-AR has not been determined, by inference from the unliganded crystal structures of other steroid receptors it appears that high affinity ligand binding to the AR causes significant conformational changes resulting in the repositioning of the critical activation function 2 (AF2) region of helix 12 across the ligand-binding pocket onto the scaffold formed by helices 3, 4 and 5 [36-39]. This interaction results in the formation of a distinct hydrophobic cleft in the surface of the receptor that is critical for receptor activity and can bind short hydrophobic LxxLL like motifs found in coregulatory proteins and in the AR-NTD (Figure 2; see below) [40-42].

Conformational maturation Following translation of the AR, conformational maturation of steroid receptors by a multi-protein chaperone heterocomplex is essential for the acquisition of ligand binding competence [43]. The specific details of this process for the AR are poorly defined, but in general, it requires at least three heat shock proteins, Hsp40, Hsp70 and Hsp90, and the co-chaperones p23 and Hop [43]. In the final stages of receptor maturation, Hsp90 becomes directly associated with the receptor LBD in a process stabilized by p23, and with one of the tetratricopeptide repeat (TPR) containing proteins which include the immunophilins FKBP51/52 and CyP40, and the protein-serine phosphatase, PP5 [43]. The interaction of the co-chaperone p23 with Hsp90 is an absolute requirement for heterocomplex stabilization of unliganded nuclear receptors [43]. Although many TPR containing proteins appear to have a similar affinity for Hsp90, they exhibit specific preferences for different steroid receptors, and may play a role in hormone action by altering the affinity and specificity for ligand. It is thought that the Hsp90-containing heterocomplex dynamically associates with steroid receptors to maintain them in a conformation that, although unstable, has a high affinity for ligand binding [43]. Following hormone binding in the cytoplasm, the Hsp90 containing heterocomplex is dissociated and the steroid receptor is rapidly translocated into the nucleus.

DNA binding In the nucleus, the AR dimerises and binds to the DNA double helix at specific DNA sequences called androgen response elements (AREs). The DBDs of steroid receptors are highly conserved cysteine-rich sequences that contain two zinc finger motifs and a short C-terminal extension that forms part of the hinge (Figure 2) [44, 45]. By inference from studies of the glucocorticoid receptor, the first zinc finger mediates DNA recognition and binds to the major groove of DNA [46, 47], while conserved amino acids in the DNA-binding domain ('D' box) of the second zinc finger mediate dimerisation between steroid receptor monomers. The recognition of specific DNA sequences by steroid receptors is determined by conserved amino acids in the 'P box' of the first zinc finger, which contact specific base pairs in consensus steroid response elements [44]. However, despite their diverse biological effects, the 'P' boxes of the closely related steroid receptors, AR, GR, MR and PR, are almost identical and the core DNA-binding domain, which includes both zinc fingers, shares up to 73% identity [45].

Response elements for steroid receptors generally consist of hexameric half-sites, separated by three nucleotides, arranged as either inverted repeats (symmetrically arranged palindromes) or direct repeats (Figure 2). Recent evidence suggests that half-sites arranged as inverted repeats may induce head-to-head dimerisation of the AR, while the polarity of direct repeats may lead to head-to-tail dimerisation [47-49]. This is in contrast to the GR, which is only able to bind to AREs arranged as inverted repeats. Receptor selectivity for particular

target sequences and alternative modes of DNA binding may be determined by sequence differences in the C-terminal extension of the DBD (Figure 2), which exhibit only an ~30% identity between steroid receptors [50]. Following binding of the first zinc finger to the major grove of DNA at the hexameric recognition site, an α-helix formed by residues in the C-terminal extension is able to enter the minor grove of DNA, binding to the hexameric half site from the opposite side [48]. Together, these various sequences mediate specific AR binding and the unique regulation of target genes by androgens [51]. Following ligand dissociation, the AR is shuttled back to the cytoplasm where it can re-associate with Hsp90 and ligand, subsequently undergoing multiple rounds of nucleocytoplasmic recycling and gene activation [52].

Recruitment of cofactors The DNA bound AR dimer recruits a multi-protein complex containing members of the basal transcription machinery (e.g. TFIIF) and additional essential proteins termed cofactors, which act to up-regulate (coactivators) or inhibit (corepressors) target gene expression [53]. The p160 family of coactivators, consisting of three related proteins, SRC-1, GRIP1/TIF2, pCIP/ACTR/RAC3/AIB1/TRAM1, are the best characterised of the nuclear receptor interacting proteins. The p160 coactivators are recruited to the ligand bound receptor where they interact directly with the hydrophobic cleft formed by AF2 residues in the LBD via a set of conserved LxxLL like motifs termed NR boxes [54]. The p160 coactivators enhance steroid receptor transcriptional activity by actively recruiting secondary coactivators such as p300/CBP and pCAF, resulting in chromatin remodelling via targeted histone acetylation and the stable assembly of the pre-initiation transcriptional complex, leading to enhanced rates of transcription initiation by RNA polymerase II [53]. In contrast, corepressors such as the related proteins SMRT (silencing mediator of retinoic and thyroid hormone receptors) and NCoR (nuclear corepressor) are thought to interact with only the apo-LBD of the steroid receptors. However, recent studies in our laboratories (Buchanan, Coetzee and Tilley, unpublishes data) demonstrate that SMRT may interact with multiple domains of the AR by ligand-dependent and ligand-independent mechanisms, and may play an important role in AR signalling. The following is a summary of key AR coregulatory molecules identified to date.

AR coactivators: (i) p160 coactivators Three related proteins (SRC-1; GRIP1/TIF2; pCIP/ACTR/RAC3/ AIB1/TRAM1) belong to this family and collectively are the best characterized of the nuclear receptor coactivators (reviewed in Rosenfeld & Glass [55]). Each has three (or in the case of SRC-1a, four) LxxLL motifs called NR boxes, which bind to the AF-2 region of all NRs. While this binding interaction is conserved among all nuclear receptors, the specific binding interaction varies. Most nuclear receptors can bind to small regions of p160 proteins containing just the NR boxes. However, the AR HBD requires the NR boxes plus an additional region of the p160 coactivators located far downstream near the CBP binding site [56]. AR and GR HBDs also prefer to bind NR box 3 of GRIP1 or NR box 4 of SRC-1a, which is located at the extreme C-terminus of one alternatively spliced isoform of SRC-1 [42]. It has recently been shown that the AR can also bind to p160 coactivators by a second mechanism: the NTD of AR (LIAF region) can bind the C-terminal region of all three p160 proteins [22, 23, 57]. In this way the p160 cofactors can function as a bridge between, and can stabilize the N- and C-termini of the AR to facilitate dimerization. More recently, phosphorylation of SRC1 by MAPK has been reported to enhance its interaction with nuclear receptors suggesting that this may also be an important step to achieve maximal target gene transcription [58]. The p160 proteins furthermore facilitate AR-mediated transcriptional activation by recruiting additional coactivators, including CBP/p300, CARM1, and possibly PCAF. CBP, p300, and PCAF can acetylate histones to help remodel nucleosomes and can acetylate other proteins in the transcription initiation complex [59]. CARM1 is a histone methyltransferase, suggesting that methylation of histones or other proteins in the transcription machinery may also contribute to transcriptional activation [60].

(ii) BRCA1 Mutations in BRCA1 have been associated with familial breast cancer susceptibility. While functions for BRCA1 in DNA repair have been proposed, it was shown that BRCA1 represses the activity of the ER [61]. However, we have recently demonstrated that BRCA1 functions as a coactivator for AR (and also for ER under most conditions) [62]. Additionally we have shown that BRCA1 interacts with the AR primarily through the LIAF region of the NTD. While the mechanism is unknown, this function of BRCA1 suggests that the AR may mediate the tumor suppressor or growth regulatory effects of BRCA1. Somatic mutations in the LIAF region of the AR potentially could abrogate such regulation.

(iii) CBP/p300 and CARM1 These factors are considered secondary coactivators normally being recruited to the preinitiation transcription complex by primary coactivators like the p160 family members (reviewed in Rosenfeld & Glass [55]). They provide necessary histone acetylation (CBP/p300) or methylation (CARM1) activities for the conversion of chromatin to transcriptional permissive local structures. As stated above evidence exists for an additional binding of CBP/p300 to the LAIF subdomain of the AR. The recruitment of these secondary coactivators might be affected by structural changes in the AR-NTD.

(iv) AR-associated (ARA) proteins Chang and colleagues [63] have identified several AR-associated proteins that interact with either the AR-LBD (ARA70/RFG/ELE1, ARA55, ARA54) or the AR-NTD (ARA24, ARA160/TMF). ARA160, an AR N-terminal interacting protein also known as TATA element modulatory

factor (TMF) cooperates with ARA70 to enhance AR activity. Another AR-NTD interacting protein, ARA24, interacts with the poly-Q tract. Binding of ARA24 is decreased by expanding the poly-Q length within AR-NTD, the latter being inversely correlated with the transcriptional activity of AR. Additional studies have demonstrated that AR and some select AR coactivators such as ARA70 or ARA54 can interact with CBP and p300/CBP-associated factors that have histone acetyl-transferase activity for assisting inchromatin remodeling. More recently, Wang *et al* [64] identified a new AR associated protein, ARA267-α, that interacts with both the AR DBD and LBD. Unlike other coregulators, such as CBP, ARA267-α had little influence on AR N-C interactions, but was able to enhance AR transactivation in a ligand-dependent manner in prostate cancer cells. ARA267-α also enhances AR transactivation with other coregulators, such as ARA24 or PCAF, a histone acetylase, in an additive manner. Collectively, these observations suggest that optimal AR transactivation in prostate cancer cells requires interaction of AR with an appropriate assembly of AR associated coregulators.

AR corepressors: (i) SMRT This corepressor was discovered in the Evans lab at the Salk Institute and acts to inhibit nuclear receptor mediated transactivation activity (reviewed in Glass & Rosenfeld [54]). We have recently shown that it also acts as a powerful inhibitor of AR signaling (data not shown). Gain of function mutations in the AR might abolish/inhibit interaction with SMRT, thereby increasing AR transactivation activity.

(ii) Cyclin D1 This protein is a required component of the CDK4 complex that plays a role in cell cycle control via phosphorylation of the retinoblastoma tumor suppressor gene [65]. Cyclin D1 possibly binds directly to the AR-NTD and inhibits AR transactivation activity [66]. It is not known exactly which regions or subdomains in the AR-NTD are necessary for cyclin D1 binding or how structural alterations in the AR might affect the binding of cyclin D1 and its AR inhibitory activity.

(iii) SHP This protein (short heterodimer partner) is an orphan receptor that lacks a DNA binding domain [67] and was recently shown to interact with the AR-NTD causing a dramatic inhibition of AR-mediated transactivation activity [68]. Apparently the inhibition is due to competition with AR coactivators like GRIP1/TIF2.

Multiple pathways of activation of the AR

Ligand-dependent activation of the AR The sequence of events in the ligand-dependent activation of the AR and regulation of androgen responsive genes is shown in Figure 3, and can be summarised briefly as follows:

i. In the absence of native ligand (DHT), immature AR is complexed in the cytoplasm to a multi-protein chaperone complex which is essential for receptor maturation and the acquisition of ligand binding competence [43],
ii. Following hormone binding, the complex dissociates and the receptor is rapidly translocated into the nucleus [52],
iii. In the nucleus, the AR dimerises and binds in the major groove of the DNA double helix at specific DNA sequences called AREs. One can speculate that at this stage the receptor dimer remains transcriptionally repressed because the large NTD is folded in such a manner that protein interaction surfaces in the AF subdomains are not assessable for binding to other proteins; this main 'inhibitory function' is achieved by the interaction of AF-1 with AF-5 plus the silenced LIAF. Access to the hydrophobic binding surface of LxxLL-like peptides of coactivators is blocked by helix 12 of the LBD. This structure is further stabilized by tight intra- or intermolecular interactions potentially between multiple regions of the NTD with AF-2 in helix 12 and/or with other regions of the LBD [41, 69, 70],
iv. Subsequent transactivation reaction depends on the relative competitive recruitment of coactivators vs. corepressors to interaction surfaces in the LBD and NTD. The binding of both types of coregulators is a consequence of, or results in conformational changes that 'opens' protein-protein interaction motifs in the AR. Coactivators additionally recruit histone acetylases and methylases, while corepressors recruit histone deacetylases to the complex resulting in chromatin decondensation (activation) and condensation (inhibition), respectively (reviewed in Rosenfeld & Glass [71]),
v. Dissociation of the AR from promoters and recycling between cytoplasm and nucleus occurs multiple times [52].

Ligand-independent activation of the AR It has recently become apparent that binding of high affinity androgenic ligands is not the only mechanism by which the AR can activate target gene sequences. There is an accumulating body of evidence demonstrating that the AR can be activated in *in vitro* systems in the absence of native ligand by growth factors (keratinocyte growth factor, insulin-like growth factor-1, and epidermal growth factor), cytokines (Interleukin-6), protein kinase-A, and by overexpression of the tyrosine kinase receptor, *HER2/neu* [9, 72-75]. The mechanism(s) that cause LIA of the AR is best understood in the case of *HER2/neu* overexpression. *HER2/neu* is a transmembrane glycoprotein member of the epidermal growth factor receptor family, and is over-expressed in carcinomas of the breast, ovary, stomach and prostate [76]. Over-expression of *HER2/neu* in androgen-responsive prostate cancer cell lines enhances AR transactivation of androgen-regulated genes such as PSA, in a ligand-independent manner, and increases cell survival during androgen deprivation [75].

HER2/neu potentially affects two phosphorylation pathways leading to LIA of AR activity. One pathway involves activation of Akt and phosphorylation of the AR at two serine residues (S213, S793) and results in suppression of androgen-induced apoptosis [55]. The other pathway that promotes LIA involves MAP kinase. Inhibitors of *HER2/neu* and MAP kinase, or [77]mutation of AR-S513 (S513A), which is located in a consensus MAP kinase phosphorylation site in the LIAF of the AR-NTD, abrogate AR transactivation activity in transient transfection assays in the presence of low concentrations of androgen [9, 74]. Recently, we identified multiple AR gene mutations (see below) in the LIAF region of the receptor in hormone refractory prostate cancer patients. One of these mutations results in the substitution of a glycine for a serine (S513G) in the same codon of the MAP kinase phosphorylation site which was mutated to an alanine in the earlier studies by Yeh *et al* [9]. Collectively, these observations suggest that structural alterations in this region of the AR-NTD may impact on ligand-independent activation of the receptor by MAPK. A significant difference between LIA of AR by growth factors and by the MAP kinase pathway is that the latter is not inhibited by casodex, suggesting that the activation of the AR is independent of the LBD. More recently, Ueda *et al* [75] have shown that LIA OF AR by IL6 involves the MAPK/STAT3 signaling pathway in LNCaP cells, and that this activity is mediated by the AR-NTD. Thus, the MAPK pathway appears to be a key mediator of LIA of the AR by both IL-6 and *HER-2/neu.*

AR polymorphisms and prostate cancer risk

AR-CAG repeat In 1992, Edward's *et al* [24] reported the allelic frequency distribution of AR-CAG repeat size in different U.S. racial-ethnic populations as part of a larger survey of genetic variation in a series of different trimeric and tetrameric tandem repeats. Among African Americans, the frequency of AR alleles with less than 22 CAG repeats was 65%, as compared to 53% in Caucasians and 34% in Asian Americans. On the basis of these observations, Coetzee and Ross [78] hypothesized that AR-CAG repeat length might be associated with the higher risk of prostate cancer in African Americans, and the intermediate and low risk in Caucasians and Asian Americans respectively, and that enhanced transcriptional activity of receptors with a shorter AR-CAG allele could promote tumorigenesis by enhancing prostatic epithelial cell turnover.

In 1995, the same investigators directly tested this hypothesis in a pilot case-control study comprising 68 prostate cancer patients and 123 control subjects [79]. In agreement with Edwards *et al*, there was a prevalence of short AR-CAG alleles in African-American vs. Caucasian and Asian controls. In addition, modest though not statistically significant enrichment of short AR-CAG alleles was observed in the Caucasian prostate cancer patients. These findings were extended in an expanded follow-up study that showed a significantly higher prevalence of short AR-CAG alleles among prostate cancer patients, especially among those with advanced disease (Table 1) [80]. In addition to our studies, Hakimi *et al* [81] identified a subgroup of patients diagnosed with advanced prostate cancer who had shorter AR-CAG repeats. Hardy *et al* [82] furthermore, demonstrated an association between age of onset and AR-CAG repeat length.

Subsequently, several well-designed matched case-control studies demonstrated an approximate 2-fold increased prostate cancer risk, decreased age of onset and/or increased risk of advanced disease for reduced AR-CAG repeat length (Table 1). Giovannucci *et al* [25] used a population selected from the Physicians Health Study that included 587 prostate cancer cases and 588 matched controls. The large sample size of this study allowed the authors to stratify cases by tumor grade and stage. A highly significant inverse correlation between AR-CAG repeat length and risk of developing prostate cancer was observed when repeat size was analysed as a semi-continuous variable. Short AR-CAG alleles also correlated with an increased risk of having advanced disease, defined as a high stage or high-grade tumor at diagnosis [25]. In another study, Stanford *et al* [83] analysed AR-CAG repeat length and prostate cancer risk in 301 prostate cancer cases and 277 matched controls [83]. They noted only a small increase in the frequency of AR-CAG alleles with less than 22 repeats in cancer patients compared with controls. Nevertheless, when AR-CAG repeat size was examined as a continuous variable, an overall age-adjusted relative odds of developing prostate cancer of 0.97 was observed for each additional CAG. More recently, Hsing *et al* [84] reported that AR-CAG alleles were significantly shorter in prostate cancer patients compared to controls among Shanghai Chinese. This study was the first to demonstrate an association in a non-Caucasian population. In a recent case-control study in an Australian Caucasian population, no association was observed between AR-CAG repeat length and prostate cancer risk, but a significant effect on the age of onset was observed [85]. In other studies (Table 1), associations between AR-CAG repeat length and prostate cancer risk were not consistently observed, possibly due to small sample sizes, population differences and/or failure to appropriately match cases and controls [86-96].

While the epidemiological studies discussed above have consistently provided evidence for an association between AR-CAG repeat length and prostate cancer risk, they did not address the molecular mechanisms underlying changes in receptor activity. As stated above, *in vitro* transient cotransfection studies have shown that AR's with longer poly-Q tracts (encoded by the polymorphic CAG repeat) have normal ligand binding

affinities but lower transactivation activities [30-33, 85]. Protein expression levels are unlikely to account for this effect since they have been found to be similar for ARs containing between 9 and 42 poly-Q repeats [22]. However, two studies have reported that AR constructs with longer repeat lengths (CAG-50 to CAG-52) are unstable and undergo accelerated degradation, potentially in a ligand dependent manner [22, 97]. The poly-Q size effect in AR transactivation activity observed in most *in vitro* studies is thought to be mediated, at least in part, through altered functional interactions with cofactors. In transient cotransfection experiments, the p160 coactivators, GRIP1, AIB1 and SRC-1 exaggerate the relative difference in AR transactivation activity with altered poly-Q length [22]. As the p160 coactivators bind to regions of the AR distinct from the poly-Q tract, this effect may be mediated by steric hindrance of p160-receptor interactions when poly-Q length is increased [22]. The RAS related G-protein, Ran/ARA24, which binds to the AR NTD in the region of the poly-Q, is an AR cofactor that appears to enhance AR activity in a poly-Q size dependent manner [98]. Given the well-described role for Ran in protein nuclear transport, it is possible that larger poly-Q tracts inhibit the efficiency of Ran directed AR nuclear import [99]. Moreover, recent observations from our laboratories (Buchanan, Coetzee and Tilley, unpublished data) indicate that the corepressor, SMRT (silencing mediator of retinoic and thyroid hormone receptors) is able to largely abolish the differences in transactivation activity mediated by different Q_n lengths. Together, these results suggest that the net AR-NTD transactivation activity may be a function of the relative amounts of coactivators vs. corepressors, and that the 'penetrance' of the poly-Q variations in the AR-NTD can be affected by the ratios of different transcriptional coregulators. Clearly, more studies are required to determine whether the effects of other cofactors that act in a cell-, promoter-, and/or AR-specific manner can be directly influenced by poly-Q length, and to determine how variation in AR poly-Q length can influence prostate cancer cell growth.

AR-GGN repeat Allelic distributions of the GGN microsatellite are significantly different among racial-ethnic groups [79], with the 23-repeat GGN allele being least prevalent amongst high-risk African-Americans (i.e., 20%) and most prevalent in low-risk Asians (i.e., 70%). This is suggestive of a protective role for this allele in prostate cancer risk. It is possible that the 23-repeat GGN allele encodes an AR containing a poly-G tract of 'optimal' length for normal receptor function in prostatic epithelial cells. While this is speculative, as it is not known whether variation in poly-G length modulates AR activity, a weak though non-significant paucity of the 23-repeat GGN allele was observed among white Caucasian prostate cancer patients compared to control subjects, suggesting that there is enrichment of putative risk alleles (i.e., non 23-repeat GGN alleles) among cases [79].

Because the AR gene is X-linked, with each male inheriting a single maternal copy, it is possible to define a putative AR prostate cancer risk allelotype of short CAG (i.e., <22 repeats) and non 23-repeat GGN. Indeed, the distribution of this allelotype has been shown to be significantly different among control subjects, with African-Americans and Asians having the highest and lowest prevalence, respectively [79]. Among white Caucasian prostate cancer patients, the <22 CAG/non 23-repeat GGN haplotype conferred a 2-fold increase in risk of prostate cancer, although statistical significance was not reached [79]. Among prostate cancer patients, a nonrandom distribution of CAG and GGN alleles was also observed; 66% of patients with a short CAG allele also had a non-23 repeat GGN allele, while only 25% of patients with long CAG alleles had a non-23 repeat GGN allele. As the CAG and GGN microsatellites are in close proximity at the AR locus, it was not surprising to find evidence of linkage disequilibrium between the intragenic markers in patient samples. In contrast, there was no evidence of linkage disequilibirim between control samples when assessed either together or by ethnicity. This indicates that in normal men, either one or both of the microsatellites are hypermutable, resulting in a random distribution of CAG and GGN alleles at the AR locus. Indeed, when the rate of mutation at the CAG microsatellite was measured using single-cell assays of sperm, an exceptionally high rate of 1-4% was observed [100]. Collectively, this data suggests that a nonrandom subset of CAG and GGN AR alleles occur in men with prostate cancer.

In 3 matched case-control studies (Table 1), a positive association between AR-GGN repeat length variation and prostate cancer risk was found [84, 101, 102]. The failure to consistently demonstrate this association in other studies (Table 1) might be due to the lack of statistical power and/or failure to appropriately match cases with controls. A more detailed assessment of the effects of the AR-GGN repeat on prostate cancer risk awaits elucidation of the effects of alterations in poly-G tract length on AR function.

AR and localized disease

The role of AR in progression of clinically localized prostate cancer has only recently been addressed [103, 104]. Henshall *et al* 2001 [103] reported that AR was expressed in more than 70% of the tumor cells in localized prostate cancer, but that there was a loss of AR immunoreactivity in the adjacent peritumoral stroma which was associated with earlier relapse after radical prostatectomy. Another study by Sweat *et al* 1999 [104] found no association between AR expression and disease progression in a highly selected cohort of tumors with a

Gleason score of 6-9. In a recent study in our laboratory, the level of AR protein in tumor foci determined by video image analysis was found to be a strong predictor of the risk of relapse following radical prostatectomy [105]. While further studies are necessary to determine how AR influences disease progression in clinically localized prostate cancer, a number of mechanisms have been identified in prostatic tumors that potentially explain the increase in levels of AR immunostaining observed in tumor cells in our study. These mechanisms include amplification of the AR gene [106], changes in the methylation status of the AR promoter and hence transcription of the AR gene [107, 108], altered stability of AR mRNA [109] and ligand-independent activation or the receptor [9, 110]. Irrespective of the mechanism, increased AR levels likely result in altered expression profiles of androgen-regulated proteins, including angiogenic factors, cell adhesion molecules and cell cycle regulators (e.g. vascular endothelial growth factor, integrins and cyclin dependent kinases and their inhibitors [111-113], which could collectively contribute to disease progression.

Hormonal therapies for metastatic prostate cancer

Due to the advent of screening with serum prostate specific antigen in the 1990s, the majority of prostate cancer cases are now diagnosed with clinically organ-confined disease. However, the assumption that earlier diagnosis would result in improved survival has not been ratified. In particular, approximately 30% of patients with organ-confined disease and 60-80% of those with extraprostatic disease who receive potentially curative local therapy will eventually relapse (reviewed in [114]). These data suggests that micro-metastases are already present at the time of diagnosis.

Although it is more than fifty years since Huggins, Stevens and Hodges [115] established that surgical castration could provide effective symptomatic relief for men with prostate cancer, targeting the androgen signaling pathway remains the predominant form of treatment for patients who are either diagnosed with or subsequently develop metastatic disease (Figure 4) [4, 5]. A myriad of therapeutic agents that target the androgen signaling pathway have been developed since the initial observation was made by Huggins and colleagues. The majority of these agents act to reduce circulating levels of androgens (luteinizing hormone releasing hormone (LHRH) agonists/antagonists such as goserelin and leuprorelin, or the oestrogens diethylstilbestrol and estramustine phosphate) or to block the action of androgens through its primary cellular target, the AR (the steroidal antiandrogen, cyproterone acetate and pure antiandrogens such as hydroxyflutamide, bicalutamide, and nilutamide; Figure 4). However, despite an initial response to androgen ablation in 80-90% of patients with metastatic disease, androgen ablation is essentially palliative and disease progression eventually ensues [5, 6]. Few patients who receive androgen ablative therapies survive for more than 5 years following initiation of hormonal therapies for metastatic disease [5, 6].

As adrenal androgens also have the potential to contribute to prostate cell growth, pure antiandrogens have been used in combination with a LHRH agonist/antagonist (ie combined androgen blockade (CAB)). In the late 1980s, Labrie and colleagues reported that MAB could prolong survival of patients with metastatic prostate cancer by about 17 months [116]. However, subsequent reports were conflicting, and currently most patients initially are treated with monotherapy, usually LHRH agonists. Indeed, a recent systematic review of CAB, encompassing 20 individual trials and more than 6000 patients, concluded that CAB results in only a modest increase in survival compared to monotherapy alone, but is more likely to be associated with adverse events and reduced quality of life [117]. Recent evidence regarding the mechanisms contributing to therapy failure (see below) suggests that combinational approaches with LHRH agonists and receptor antagonists cannot completely abrogate androgen action, and may select for cells with a growth state permissive for a particular hormonal environment. The potential clinical importance of a therapy-mediated selective pressure is illustrated by the syndrome of steroid-hormone and anti-antiandrogen withdrawal, which is characterized by tumor regression and decreasing serum levels of PSA when treatment with an antiandrogen, progestational agent or estrogen is selectively discontinued at a time of clinical progression [118]. A withdrawal response has been observed in up to 30% of patients with hormone refractory prostate cancer when treatment with the antiandrogen, hydroxyflutamide is terminated [118-120], and has also been documented following withdrawal of the AR antagonists, nilutamide and bicalutamide, the estrogens, diethylstilbestrol and megestrol acetate, and the progestational agent, chlormadinone acetate [121-126]. Withdrawal responses have been reported at a higher incidence following combined therapy, consisting of castration or LHRH agonists in combination with an AR antagonist, compared to antagonist alone, leading the authors to conclude that prolonged exposure to antiandrogens was the predominant factor in the withdrawal response rather than a low level of androgens [122, 127]. In one study, inhibition of adrenal steroid production with ketoconazole following discontinuation of antiandrogen therapy resulted in a higher proportion of patients (55%) exhibiting a withdrawal response and an increased duration of response [128] than reported for withdrawal of the antiandrogen alone [127].

Recent evidence suggests that resistance to androgen ablation therapies is not necessarily due to loss of androgen sensitivity, but may develop as a consequence of a deregulated androgen-signaling axis resulting from (i) amplification and /or altered expression of the AR, (ii) mutation of the AR gene, (iii) inappropriate interaction with AR co-regulatory molecules (co-activators, co-repressors), or (iv) ligand-independent activation of the AR by growth factors and cytokines [7-9] (reviewed in [10-12]). In addition, many somatic genetic alterations

implicated in prostate cancer initiation and progression may also have a direct effect on androgen signaling in prostate cells (Buchanan, Tilley & Coetzee, unpublished observations).

AR and the failure of androgen ablation therapies

AR levels Initial studies using both androgen-unresponsive Dunning rat adenocarcinoma and human prostate cancer cell lines suggested that loss of AR mRNA and protein could be a mechanism to explain the failure of androgen ablation therapies [129, 130]. Subsequent immunohistochemical studies of clinical prostate cancer demonstrated that the AR is expressed in essentially all metastatic tumors, including those that continue to grow following androgen ablation [131-137]. Other studies have shown that the AR in recurrent, hormone refractory prostate cancer, is expressed at similar levels to those in androgen-dependent prostate tumors [138]. Recent studies using the recurrent CWR22 xenograft model and its derived cell line, CWR-R1, as well as the LNCaP C4-2 cell line derived from LNCaP cells after prolonged periods of culture in the absence of androgen, suggest that the AR is expressed at similar levels in both androgen dependent and recurrent tumors, but is more stable in recurrent tumors in the absence of androgen [139]. In addition, the concentration of androgen required for stimulation of the CWR-R1 and LNCaP C4-2 cell lines was 4-fold lower than that required for androgen-dependent LNCaP cells. This concentration of DHT is comparable to the levels in prostates of men treated with androgen ablation. The observations that AR is expressed at relatively high levels in recurrent CWR22 human tumor xenografts and cell lines and is hypersensitive to low levels of androgens, suggest that androgen-signaling and the associated activation of androgen-regulated genes in human prostate tumors is sufficient to maintain tumor growth following androgen ablation. More recently, Kim *et al* [140] reported a marked reduction in AR expression in CWR22 tumors two days post-castration, but subsequent re-expression of AR and androgen-regulated genes in recurrent tumors 150-days post-castration to levels comparable to those observed in tumors from intact mice. Reactivation of AR expression was associated with renewed proliferation of tumor cells at 120–150 days post-castration, suggesting that this is a critical factor contributing to renewed tumor growth.

Amplification of the AR gene, which also has the potential to contribute to increased AR protein levels, has been reported in 22% of prostate cancer metastases [141], and in 23-28% of primary tumors following androgen deprivation [106, 142]. An average 2-fold increase in the levels of both AR and PSA proteins has been reported in prostate tumor samples with AR gene amplification compared to samples where no AR amplification was found [7, 143]. Increased AR protein levels in metastatic prostate tumors may also augment the sensitivity of the androgen-signaling axis, thereby contributing to disease progression during the course of androgen ablation. In addition, AR gene amplification and overexpression of the receptor have been reported in hormone refractory prostate cancer following monotherapy, but not in primary prostate tumors [7], suggesting that hormonal therapy could select for cells with an ability to maintain growth during treatment.

AR gene mutations The first indication that AR gene mutations might contribute to the failure of androgen ablation therapies came from studies of the androgen responsive human prostate cancer cell line, LNCaP. The AR in LNCaP cells contains a single amino acid substitution (Thr-Ala877) that facilitates inappropriate activation by glucocorticoids, progestins, adrenal androgens, estradiol, and the anti-androgen hydroxyflutamide [144, 145]. Subsequently, somatic missense mutations have been detected throughout the AR coding sequence at frequencies of up to 50% in advanced primary tumors and metastatic deposits [146-149] (reviewed in [10]). These mutations consistently result in receptors that exhibit decreased specificity of ligand-binding and enhanced receptor activation by androgens and non-classical ligands compared to wild type AR (wtAR; reviewed in [150, 151]). More recently in collaboration with Dr Norman Greenberg at Baylor College of Medicine, Houston, TX, we reported the identification of AR gene mutations in the autochthonous transgenic adenocarcinoma of mouse prostate (TRAMP) model [152]. Analogous to the findings in clinical prostate cancer, AR gene mutations detected in TRAMP tumors also result in receptors that contribute to altered androgen signaling [152]. In the TRAMP model, different hormonal environments result in the selection of AR variants with mutations in distinctly different regions of the receptor [152]. All of the missense AR gene mutations (6/6) identified in tumors derived from intact TRAMP mice at 24-28 weeks of age were located in the LBD (Figure 5) [152]. In contrast, most of the missense mutations (7/9) identified in mice castrated at 12 weeks of age were located in the AR-NTD. Four of the mutations identified in castrated TRAMP mice resulted in receptors with increased transactivation function in the absence of ligand. Selection for AR gene mutations with a phenotype permissive for growth is also evident from recent studies of clinical prostate cancer. In one study, AR gene missense mutations were detected more frequently in patients who were treated with combined androgen blockade using hydroxyflutamide (5/16) compared to monotherapy (1/17) with androgen ablation [148]. The same mutation in codon 877 (T877A) was found in all five of the patients treated with CAB. A different mutation resulting in a D890N substitution in the AR was identified in the patient treated with monotherapy. Whereas no difference in activation of the D890N variant and wtAR was observed, functional studies demonstrated that the T877A mutations resulted in receptors exhibiting a marked increase in activity in response to hydroxyflutamide, but not to DHT or other androgenic

ligands, suggesting that flutamide treatment selects for tumor cells expressing a hydroxyflutamide-inducible AR variant [148]. More recently, Haapala and colleagues [153] reported the identification of AR gene mutations in 36% (4/11) of tumors from patients treated with CAB. These mutations are in different regions of the AR to those reported in codon 877 by Taplin *et al* [148], suggesting that different types of AR variants may be specifically selected for by different treatments. These data support the hypothesis that AR gene mutations identified in prostate cancer provide a selective growth advantage given the appropriate hormonal environment, resulting in the re-emergence of tumor growth during the course of hormone ablation therapy.

Structural and functional collocation of AR variants We recently reported that nearly 80% of missense AR gene mutations identified in clinical prostate cancer cluster to discrete regions of the receptor that collectively span less than 15% of the coding sequence (Figure 5) [151].

(i) Ligand-binding domain variants In the LBD, mutations collocate to (i) the 'signature sequence', a conserved twenty amino acid region of nuclear receptors involved in ligand recognition and specificity [154], (ii) AF-2, a binding site for the p160 cofactors, and (iii) a region at the boundary of the hinge and LBD containing a four amino acid tetrapeptide (^{668}QPIF^{671}) that may define a protein-protein interaction surface (Figure 5). Many of the AR gene mutations identified in the LBD of the AR in the TRAMP model, xenografts and cell lines occur in the same three regions as mutations in clinical prostate cancer (Figure 5). For example, a Phe-Ile671 mutation identified in an intact TRAMP mouse collocated to the ^{668}QPIF^{671} tetrapeptide with mutations identified human prostate cancer [8]. AR gene mutations identified in both clinical prostate cancer and TRAMP tumors in this region exhibit a 2-4 fold greater transactivation activity in response to DHT, non-classical ligands and hydroxyflutamide compared to wtAR [8], without altering ligand-binding kinetics, receptor levels or DNA binding capacity. Homology modeling revealed that the ^{668}QPIF^{671} tetrapeptide residues form a potential protein-protein interaction surface that is markedly disrupted by the naturally occurring mutations, providing a mechanism that could explain the observed gain in transcriptional activity [8].

Another AR missense mutation identified in the TRAMP model, Phe-Ser697, is located adjacent to the signature sequence. The Ser697 AR variant exhibits markedly reduced transactivation responses to progesterone and 17ß-estradiol, but enhanced response to R1881 compared to wtAR [152]. These results are consistent with the role of the signature sequence in ligand recognition and specificity, and with previous reports of mutations in this region in clinical disease (reviewed in [10, 151]). Analysis of the Thr-Ala877 AR variant, identified in a significant proportion of clinical prostate tumors and in the human prostate cancer cell line, LNCaP, has confirmed that this mutation exhibits increased transactivation activity in response to progesterone, 17β-estradiol, adrenal androgens and hydroxyflutamide compared to wtAR [155]. The recently determined AR-LBD crystal structure [36, 37] allowed us to use homology modeling to demonstrate that this mutation results in changes to the shape and volume of the ligand binding pocket such that bulkier ligands like progesterone can be accommodated [152].

(ii) DNA binding domain variants In addition to the above observations, five somatic missense mutations have been identified in clinical prostate tumors that collocate to a 14 amino acid region at the carboxyl-terminal end of the first zinc finger motif in the DNA binding domain (DBD) of the AR [146, 149]. The effect of each of these mutations is unknown, but none of the codons in which they occur have been reported to contain mutations that cause receptor inactivation in the clinical syndrome of androgen insensitivity. Mutations in the AR-DBD have been shown to selectively affect transactivation and transrepression functions of the AR on different promoters despite a reduced DNA binding ability [156, 157], and may represent a predisposing factor for male breast cancer [158]. Due to the high homology of the DBD across members of the nuclear receptor superfamily, the cell and promoter specificity of different receptors is, in part, mediated by only a few changes in DBD sequence [159]. It has been speculated that mutations in the DBD could result in AR variants that bind to response elements normally specific for other nuclear receptors [158], leading to inappropriate activation or repression of growth regulatory pathways. In an analogous manner, mutations in androgen receptor response elements have been shown to increase the sensitivity of the enhancer for the glucocorticoid receptor [160]. Modeling experiments suggest that residues in the AR-DBD could form a protein interaction surface [158], and several AR coactivators that interact with the DBD in a ligand dependent manner [161-163] are predicted to alter receptor activity via local chromatin remodeling [161], interaction with components of the transcriptional machinery [162], or inhibiting nuclear export [164]. It is also possible that mutations in the DBD of the AR gene identified in prostate cancer could alter the affinity of receptor binding to response elements, resulting in altered expression of a range of target genes regulated by the AR.

(iii) Amino-terminal transactivation domain variants Nearly half of the AR gene mutations identified in clinical prostate cancer are located in the amino-terminal transactivation domain (NTD) of the receptor. AR gene mutations in this domain have also been identified in the TRAMP model and, analogous to the observations for

the LBD, these mutations cluster with those identified in clinical prostate cancer to discrete regions within the NTD that are implicated in receptor function. The main regions of collocation in the NTD are (i) within and adjacent to the poly-Q tract (codons 54-78), which as discussed above has been implicated in modulating receptor activity and prostate cancer risk, and (ii) a region amino terminal to the DNA-binding domain (codons 502-535) known to modulate the transactivation capacity of the receptor in both a ligand-dependent and ligand-independent manner (ie a ligand-independent activation function or LIAF; Figure 5) [165, 166].

Somatic contractions in the CAG repeat of the AR gene, which potentially increase AR activity in a sub-population of cells and thereby contribute to disease progression, have been identified in three independent studies of clinical prostate tumors [167-169]. In addition, we have recently identified a somatic mutation within the CAG repeat of the AR gene in a primary prostate tumor that results in interruption of the polyglutamine repeat by 2 leucine residues. This AR variant has a 2-4 fold greater ability to transactivate target genes compared to wtAR in the presence of physiological concentrations of DHT [170]. Four additional somatic mutations have been identified in or adjacent to the CAG repeat region of the AR gene in human prostate cancer [146], but have not yet been characterized. Further analysis of inherited and somatic alterations in this region of the AR gene in prostate cancer is warranted to determine the contribution of this motif to AR activity, and its potential to influence the development and/or progression of the disease.

A second region of the NTD where amino acid substitutions have been identified in hormone-refractory prostate tumors is the LIAF. In a recent study, we examined the complete coding sequence of the AR gene for mutations in metastatic tissue biopsies from 12 patients who exhibited the clinical syndrome of steroid-hormone and anti-antiandrogen withdrawal response to hydroxyflutamide. Five mutations identified in this study, collocated with a previously identified mutation [146], to a small carboxyl-terminal portion (aa 502-535) of the NTD of the AR. An additional mutation (Asp-Gly526) previously identified in our studies of primary prostate tumors [146], and another identified in the TRAMP model [152], collocate to this region of the AR. This carboxyl-terminal region of the amino-terminal domain of the AR is known to modulate the transactivation capacity of the receptor in both a ligand-dependent and ligand-independent manner [165, 166], and has recently been shown to be involved in direct interactions with the p160 coactivators and the transcription regulator p300/CBP [22, 23, 171]. This region of the NTD also contains the binding site for the receptor accessory factor (RAF), which enhances the specific DNA binding of rat AR [172]. Further evidence in support of the LIAF region being important in AR transactivation has been provided by recent studies demonstrating that mutation of S513, which is located in a consensus MAP kinase phosphorylation site, partially inhibits ligand-independent activation of the receptor by HER-2/neu [9]. Collectively, these observations suggest that the LIAF region may contain an interaction surface for accessory proteins that promote ligand-independent transactivation. Mutations in this region may alter the ability of the receptor to respond to these and other coregulatory proteins, thereby altering the transactivation capacity of the AR in a manner that could provide a growth advantage to prostate cancer cells in an appropriate hormonal environment.

Altered interaction with co-regulatory molecules Gregory *et al.* [173] have shown that overexpression of the p160 coactivators, TIF2/GRIP1 and SRC-1, observed in recurrent tumors from CWR22 human prostate xenografts and clinical prostate cancer, increases AR transactivation capacity at physiological concentrations of non-classical ligands (adrenal androgens, estradiol and progesterone). Overexpression of p160 coactivators, especially TIF2, favors an interaction between the coactivator and AF2 in preference to formation of an N-C interaction (between an LxxLL-like motif in the NTD and a hydrophobic interaction surface that overlaps with AF-2). Overexpression of TIF2 could therefore result in the recruitment of TIF2 to AF-2 by low affinity steroids resulting in the inhibition of N-C interaction and the derepression of the LIAF in the NTD.

Characterization of the functional relationship between the AR and AR associated coregulators by Chang and colleagues [174, 175] has shown that ARA70 and ARA55 can enhance the androgenic effects of 17β-estradiol and hydroxyflutamide, the latter an antiandrogen commonly used in the treatment of metastatic prostate cancer. ARA70 can enhance the androgenic activity of 17β-estradiol and antiandrogens toward AR, suggesting that specificity of sex hormones and agonist activity of antiandrogens can be modulated by different coregulators. Their studies also demonstrated that ARA70, ARA55 and ARA54, but not steroid receptor coactivator-1 (SRC-1) and Rb, could significantly enhance AR mediated transactivation by androst-5-ene-3β,17β-diol, a precursor to testosterone. Thus, it is possible that the specificity and sensitivity of sex hormones and antiandrogens for activating the AR can be selectively modulated by AR coactivators, thereby contributing to the growth of prostate tumors in the presence of low concentrations of endogenous androgenic steroids or non-classical ligands.

Characterization of a Glu-Gly236 substitution identified in a TRAMP tumor revealed that the variant receptor had increased transactivation function compared to wtAR in response to R1881 and 17β-estradiol only in the presence of the coactivator, ARA70, and increased response to R1881 but not 17β-estradiol in the presence of the coactivator, ARA160 [152]. In addition, recent studies suggest that phosphorylation of cofactors (e.g. SRC1)

by MAPK could alter the transactivation activity of the AR by more effectively recruiting the cofactor to the basal transcription complex [58], or by bridging AF1/AF5 in the NTD with AF2 thereby promoting an N-C interaction and a maximal functional response. Collectively, these findings suggest that the phenotype of some AR gene mutations may only be apparent in the presence of the appropriate milieu of coregulators. All of the mechanisms discussed above theoretically could result in enhanced AR transactivation activity *in vivo* in the presence of low concentrations of DHT or in the absence of circulating levels of native ligand, thereby contributing to the development of resistance to androgen ablation therapies used in the treatment of metastatic prostate cancer. These findings also suggest that disruption of the interaction between the AR and AR specific cofactors may serve as a novel therapeutic target in the treatment of metastatic prostate cancer.

Ligand-independent activation of the AR Another potentially important mechanism contributing to the failure of androgen ablation is ligand-independent activation (LIA) of the AR (see above). Aberrant expression of growth factor receptors also contributes to development and progression of prostate cancers [10, 151]. HER2/*neu* (c-erbB-2), a transmembrane glycoprotein member of the epidermal growth factor receptor family, is overexpressed in carcinomas of the breast, ovary, stomach and prostate [176-179]. Unlike other epidermal growth factor receptor members, HER2/*neu* has intrinsic tyrosine kinase activity and mediates signal transduction in the absence of ligand [180]. Recent studies suggest that HER2/*neu* expression is increased in hormone refractory prostate tumors compared to earlier stages of disease [76, 181]. Over-expression of HER2/*neu* in androgen-responsive prostate cancer cell lines enhances AR transactivation of androgen-regulated genes such as PSA, in a ligand-independent manner, and increases cell survival during androgen deprivation [9, 74]. Although the mechanism involved in HER2/*neu* modulation of AR transactivation has not been fully characterized, HER2/*neu* expression is associated with activation of MAP kinase and Akt (protein kinase B) pathways which have been implicated in ligand-independent activation of the AR [9, 110]. Tumor cells with increased HER2/*neu* expression and high AR may have a selective growth advantage. For example HER2/*neu* activation of an Akt-AR pathway [9, 74, 110] may confer a clonal advantage by promoting cancer cell survival via the androgen signaling axis [110] or by the induction of Akt-dependent pathways [180]. In a recent study using prostate cancer xenograft models, Herceptin (a monoclonal antibody directed against activated HER2/*neu*) monotherapy resulted in antiproliferative activity in androgen-dependent LNCaP and CWR22 tumors, but no significant growth inhibition was observed in androgen-independent CWR22 tumors [182]. These observations suggest that signaling through the AR is a requisite for a response to Herceptin in prostate cancer [182]. Thus, patients with elevated levels of both HER2/*neu* and AR immunostaining may benefit from early treatment targeting both the AR and HER2/*neu* signaling cascades.

Conclusions

AR signaling plays a key role in many phases of prostate cancer biology. Carefully matched case-control and other studies suggest that length variation of the AR-CAG and AR-GGN polymorphic microsatellite repeats contributes to prostate cancer risk, and may also influence age of onset and tumor pathology, by altering AR transcriptional activity and/or interaction with AR coregulators. In addition, the consistent finding that expression of the AR is maintained in virtually all advanced prostate tumors, and that increased expression of and/or gain of function mutations in the AR gene occur frequently in clinical disease, has provided compelling evidence that the failure of androgen ablation therapy for metastatic prostate cancer does not result from a loss of androgen signaling, but rather the acquisition of genetic changes that lead to aberrant activation of the androgen signaling axis. Other mechanisms potentially contributing to alterations in AR signaling in prostate cancer include inappropriate interaction with coregulatory proteins (eg between p160 cofactors and AF-2 to alter the specificity of activation of the receptor) and ligand-independent activation of the AR by growth factors, receptor tyrosine kinases and cytokines. Thus, resistance to androgen ablation and survival of prostate cancer cells is not necessarily due to the evolution of a growth state that circumvents the androgen-signaling axis, but could be explained in part by increased activity of the AR in the presence of either native or alternative ligands, or cross-talk with other signaling pathways. This provides a unifying hypothesis for the failure of conventional androgen ablation therapies used in the treatment of metastatic prostate cancer, and has important implications for the development of novel therapeutic interventions for hormone refractory disease.

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Figure 1: AR gene and protein structure. A. Schematic representation of the AR gene on chromosome Xq11-12 showing important binding sites for SRY and SP1 transcription factors. Individual exons are separated by up to 16kb of intronic sequence. **B**. AR mRNA transcript showing alternative splice and polyadenylation sites. Translation is primarily directed from the first of two initiating methionine residues. **C**. Structure of the predominant (AR-B) form of the AR. Indicated are the amino-terminal transactivation domain (NTD), DNA-binding domain (DBD), hinge region (H), ligand-binding domain (LBD), and the ligand-dependent activation function, AF2. **D**. The structure of the NTD showing the position of the two polymeric amino acid stretches (polyglutamine, Qn; polyglycine, Gn), ligand-independent activation function (LIAF), constitutive activation functions (AF1 and AF5), and the position of the two 'LxxLL-like' motifs implicated in interaction between the NTD and LBD. Structures are not to scale.

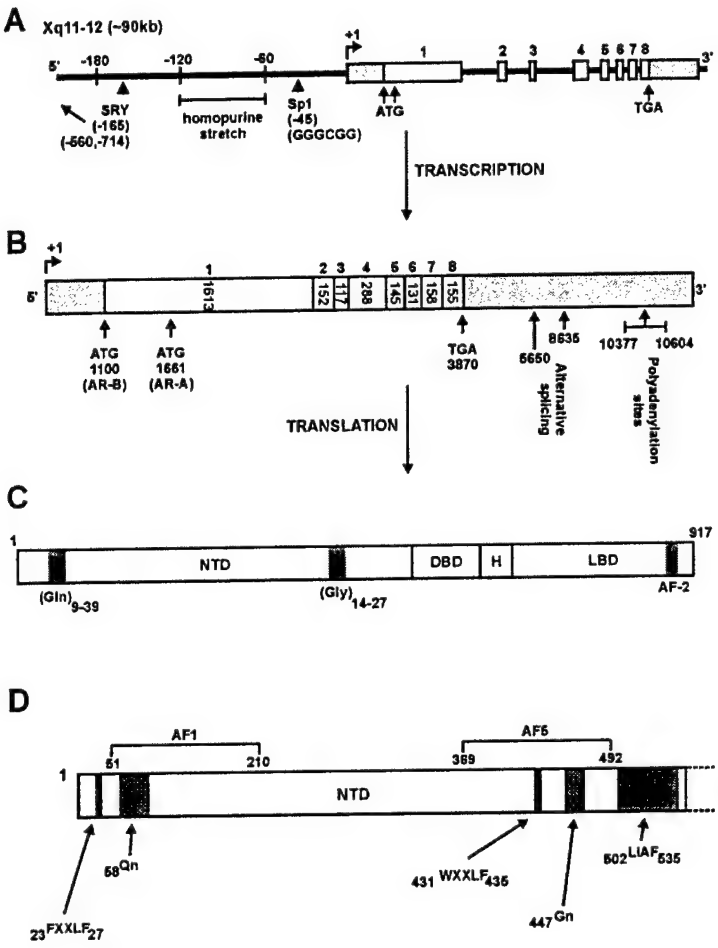
Figure 2: LBD and DBD structure. A. Ribbon diagram of the AR-LBD based on the published crystal structure showing alpha-helix and beta-sheet with the LXXLL peptide of the p160 coactivator, GRIP1, modelled into the hydrophobic cleft formed by AF-2. **B**. Topology and electrostatic surface potential diagram of the AR-LBD showing position of the bound LXXLL peptide of GRIP1. Homology modelling of the AR (A, B) was performed by Dr Jonathan Harris, Queensland Institute of Technology. **C**. The AR DBD consists of a highly conserved cysteine-rich sequence that contain two zinc finger motifs and a short C-terminal extension (CTE) that forms part of the hinge. The first zinc finger mediates DNA recognition via the highly conserved 'P box' and binds to the major groove of DNA, while conserved amino acids in the 'D box' of the second zinc finger mediate dimerisation between steroid receptor monomers. Sequences in the CTE bind to the minor groove of DNA on the opposite side of the helix to the first zinc finger and mediate sequence specificity of steroid receptors. Indicated are amino acids that determine the specificity of DNA binding (*), and those likely to make base-pair contacts in the hormone response element (HRE) half-site (#). **D**. Known androgen response elements (AREs). **a**. Consensus glucocorticoid response element. **b**. AREs that have an inverted repeat structure consisting of imperfect palindromes bind both AR and GR. **c**. AREs that bind AR selectively have a partial direct repeat structure. HRE, hormone response element; PB, rat probasin gene; sc, human secretory component gene; slp, mouse sex limited protein gene.

Figure 3: Mechanism of AR activation by ligand-dependent pathways. Following synthesis, the AR exists in dynamic equilibrium between an immature state and an active form capable of binding high affinity androgenic ligands via association/dissociation with a complex that includes heat-shock proteins, p23 and a tetratricopeptide (TPR) containing protein. Ligand binding results in the dissociation of this complex, receptor dimerisation and phosphorylation, nuclear transport, DNA binding, the recruitment of components of the transcription machinery (TM) and other cofactor molecules, such as the p160 coactivators, and ultimately, the activation of androgen-regulated gene pathways.

Figure 4: Androgen signaling axis in prostate cancer: androgen ablation therapies The growth and development of the normal prostate requires a functioning androgen signaling pathway, which originates with the hypothalamus/pituitary axis. The continued reliance of metastatic prostate cancer on androgen signaling for growth is exploited in androgen ablation therapies, which aim to either reduce the circulating level of androgens or block their action in the prostate. Lines represent the path of the normal signaling molecules (as shown), while dark bars represent the molecular or cellular target of agents (as indicated) that have been used for treatment of prostate cancer. Combined androgen blockade, which uses a combination of LHRH analogues and AR antagonists, is often used for metastatic disease that has progressed on monotherapy.

Figure 5: Collocation of AR gene mutations in prostate cancer. The majority (80%) of AR gene mutations identified in clinical prostate cancer collocate to discrete regions within the receptor NTD and LBD as indicated. Mutations in each of these regions (polyglutamine repeat, PolyQ; ligand-independent activation function, LIAF; a tetrapeptide at the boundary of the hinge and LBD, QPIF; signature sequence, SS; activation function 2, AF2) have been shown to result in changes to receptor activity (as shown) that could explain the outgrowth of prostate cancer cells containing these mutations during androgen ablation therapies. The position of mutations identified in human prostate cancer cell lines, xenografts and the transgenic adenocarcinoma of the mouse prostate model (TRAMP) is indicated. Hormonal treatment of TRAMP mice drives the selection of AR gene mutations to either the NTD (7/9 castrate mice versus 0/6 in intact animals) or the LBD (6/6 intact mice versus 2/9 castrate).

Figure 1: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(AR-A)
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3870
5650
8635
Alternative
splicing
10377
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Polyadenylation
sites
TRANSLATION
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917
NTD
DBD
H
LBD
AF-2
(Gln)9-39
(Gly)14-27
D
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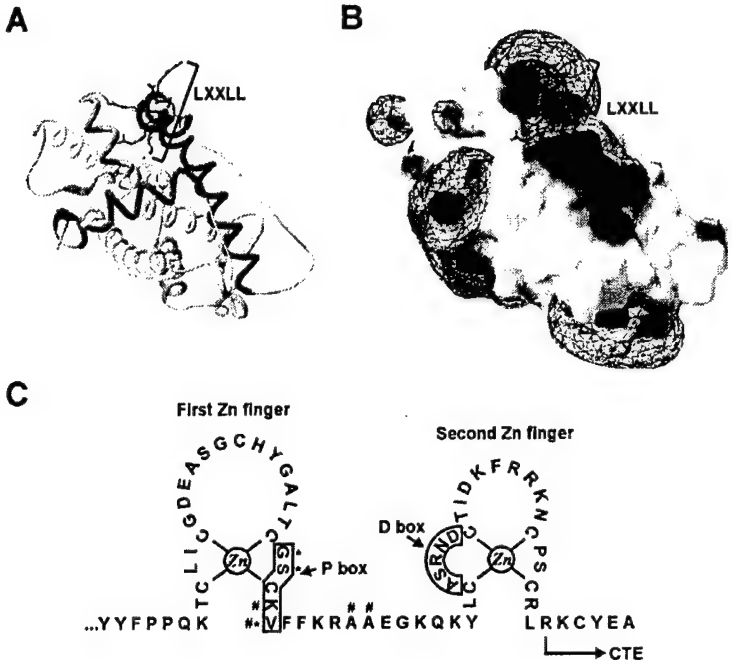
Figure 2:



D

a. Consensus glucocorticoid response element

	GGTACA	nnn	TGTTCT

b. Inverted repeats

C3(1)ARE	AGTACG	TGA	TGTTCT
PB-ARE-1	ATAGCA	TCT	TGTTCT

c. Direct repeats

PB-ARE-2	GGTTCT	TGG	AGTACT
sc-ARE1.2	GGCTCT	TTC	AGTTCT
slp-HRE2	TGGTCA	GCC	AGTTCT
CONSENSUS	KGNTCW	nnn	AGTWCT

Figure 3:

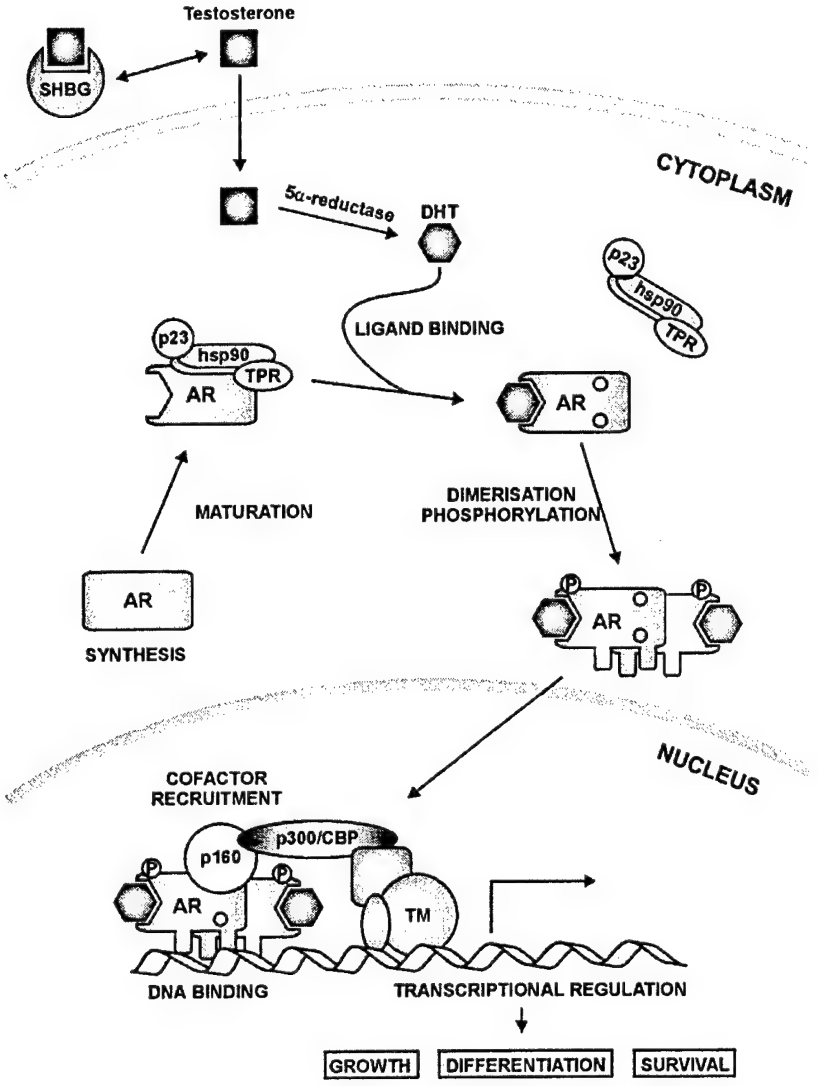

Testosterone
SHBG
CYTOPLASM
5α-reductase
DHT
p23
hsp90
TPR
LIGAND BINDING
AR
MATURATION
DIMERISATION
PHOSPHORYLATION
SYNTHESIS
NUCLEUS
COFACTOR
RECRUITMENT
p300/CBP
p160
TM
DNA BINDING
TRANSCRIPTIONAL REGULATION
GROWTH
DIFFERENTIATION
SURVIVAL

Figure 5:

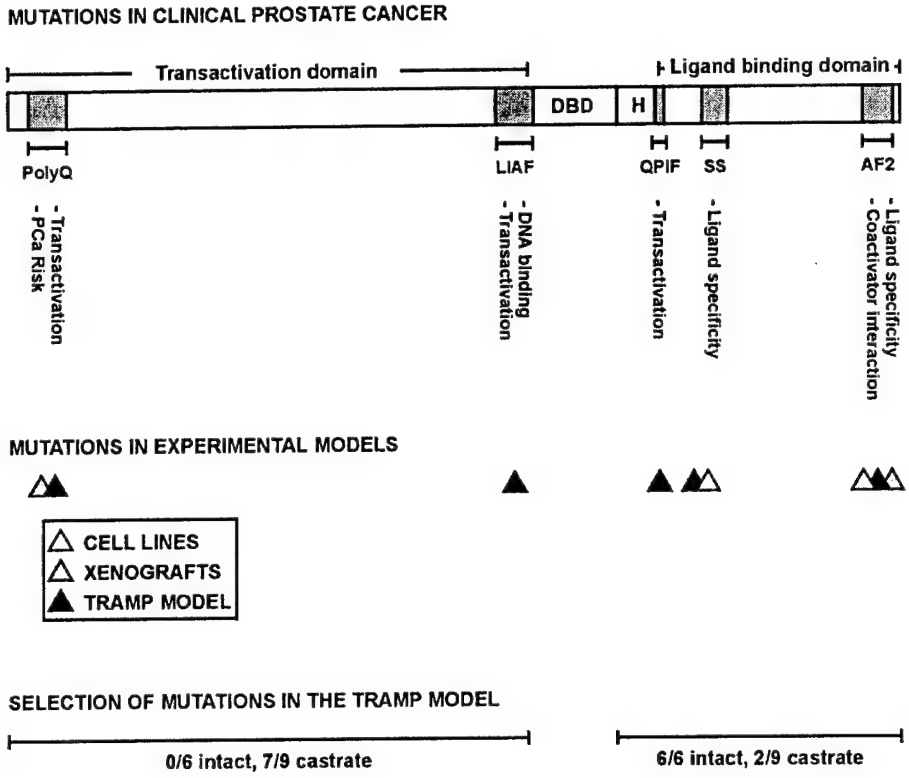

MUTATIONS IN CLINICAL PROSTATE CANCER
Transactivation domain
Ligand binding domain
DBD
H
PolyQ
LIAF
QPIF
SS
AF2
- Transactivation - PCa Risk
- DNA binding - Transactivation
- Transactivation
- Ligand specificity
- Ligand specificity - Coactivator interaction
MUTATIONS IN EXPERIMENTAL MODELS
CELL LINES
XENOGRAFTS
TRAMP MODEL
SELECTION OF MUTATIONS IN THE TRAMP MODEL
0/6 intact, 7/9 castrate
6/6 intact, 2/9 castrate

Table 1. Associations between AR CAG and/or GGN microsatellites and prostate cancer risk, nature of disease at diagnosis and age at onset.

Study reference	Subjects	AR CAG repeat associations			AR GGN repeat associations		
		risk	stage/grade	age at onset	risk	stage/grade	age at onset
Pilot studies							
Irvine et al., 1995 [79]	US Caucasian	yes	N/A	N/A	yes	N/A	N/A
Hardy et al., 1996 [82]	US Caucasian	N/A	no	yes	N/A	N/A	N/A
Ingles et al., 1997 [80]	US Caucasian	yes	yes	N/A	N/A	N/A	N/A
Hakimi et al., 1997 [81]	US Caucasian	yes	yes	no	yes	no	no
Matched case-control studies							
Giovannucci et al., 1997 [25]	US Caucasian	yes	yes	no	N/A	N/A	N/A
Stanford et al., 1997 [83]	US Caucasian	yes	no	yes	yes	no	yes
Platz et al., 1998 [102]	US Caucasian	N/A	N/A	N/A	yes	N/A	N/A
Hsing et al., 2000 [84]	Chinese	yes	no	no	yes	no	no
Beilin et al., 2001 [34]	Australian White	no	no	yes	N/A	N/A	N/A
Other studies							
Ekman et al., 1999 [86]	Swedish White	yes	N/A	N/A	N/A	N/A	N/A
Edwards et al., 1999 [87]	British Caucasian	no	no	N/A	yes	no	N/A
Correa-Cerro et al., 1999 [88]	French/German White	no	no	no	no	no	no
Bratt et al., 1999 [89]	Swedish White	no	yes	yes	N/A	N/A	N/A
Lange et al., 2000 [90]	US Caucasian (high risk)	no	no	no	N/A	N/A	N/A
Nam et al., 2000 [91]	Canadian	N/A	yes	N/A	N/A	N/A	N/A
Latil et al., 2001 [92]	French White	no	no	yes	N/A	N/A	N/A
Modugno et al., 2001 [93]	US Caucasian	yes	N/A	N/A	N/A	N/A	N/A
Miller et al., 2001 [94]	US Caucasian	no	N/A	N/A	no	N/A	N/A
Panz et al., 2001 [95]	S. Africans (Black & White)	yes	yes	N/A	N/A	N/A	N/A
Cude et al., 2002 [96]	unknown US population	N/A	yes	no	N/A	N/A	N/A

N/A, denotes not applicable or not assessed; Yes, association between polymorphism and listed parameter; No, no significant association detected between polymorphism and listed parameter.

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Title: The AR CAG repeat and Prostate Cancer risk.

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The androgen receptor (AR) gene comprises eight exons located at chromosome Xq11-12 and encodes an mRNA transcript of approximately 11 kb.[1-5] Situated within exon 1 of the AR gene is a polymorphic CAG trinucleotide repeat which encodes a polyglutamine (poly-Q) tract of variable length in the N-terminal domain of the AR protein. The normal size range of this poly-Q tract is between 6 and 39 repeats.[6, 7] The gene is considered a good candidate to play a role in the etiology of prostate cancer because of this variation and the well-documented importance of AR signaling in prostate development and tumor growth. Although earlier family studies failed to link prostate cancer to the AR locus, the CAG repeat variations of this locus have been considered to play a 'modifying' role in prostate cancer risk (as apposed to causing it). The AR on the X chromosome is, furthermore, thought to contribute to the increased brother/brother vs. father/son risk associations observed in several studies.[8-10] More recently, however, linkage to the AR locus (LOD score 3.06, p=0.00053) was observed in 254 families if, and only if, Gleason score, age of onset, male-to-male transmission, and/or number of affected first-degree family members were included as covariates.[10]

The evolution of the AR CAG repeat: Over the course of primate evolution the AR CAG microsatellite has progressively expanded.[11, 12] For example, it has been shown that the mean uninterrupted CAG repeat size in Old world marmoset, macaque, and drill monkeys is 3, 7, and 8 respectively.[11] Furthermore, several other human microsatellite repeats are longer than in the equivalent genes in primates, suggesting a selection bias towards expansion of these microsatellite repeats over the course of human evolution.[13] Any such mutational bias and resulting directional expansion of the microsatellite repeats

would, of course, be expected to be evolutionarily tolerated only so long as these changes did not result in a significant decrease in reproductive fitness, such as a functional disruption of the protein.

The AR CAG repeat and Kennedy's disease: Abnormal expansion of the CAG microsatellite to 40 repeats or more results in a rare, X-linked, neurodegenerative disorder in adults called spinal and bulbar muscular atrophy (SBMA), also known as Kennedy's disease.[14, 15] The hallmark of the disease is progressive muscle atrophy secondary to progressive loss of motor neurons in both the brain stem and spinal cord. In addition, men frequently develop symptoms consistent with partial androgen insensitivity, including gynecomastia and testicular atrophy, which are indicative of aberrant AR function.[16, 17] In support of this, *in vitro* transient cotransfection experiments have demonstrated that the mutant AR encoded for in SBMA has reduced transactivation activity when compared with wild type AR, while maintaining normal androgen-binding affinities.[18, 19] Numerous studies have subsequently demonstrated an inverse relationship between CAG repeat length and AR transactivation activity over a wide range of CAG repeat sizes that encompass the entire range of normal AR alleles.[20-23] Thus, the abnormal AR poly Q tract expansion seen in men with SBMA results in a loss of AR transactivation competency, which manifests itself as partial androgen insensitivity. Androgen receptors with the smaller poly Q tract in "normal" men can then, in effect, be viewed as having a gain of function through an increase in transactivation activity, and as a consequence result in an apparent increase in androgen sensitivity.

The AR CAG repeat and prostate cancer risk: In 1992 Edward's et al.[6] reported on the allelic frequency distribution of AR based on the CAG repeat size in different U.S. racial-ethnic populations as part of a larger survey of genetic variation in a series of different trimeric and tetrameric tandem repeats. This study found that among African Americans, who are at a higher risk for developing prostate cancer, the frequency of AR alleles with less than 22 CAG repeats was 65%, as compared to 53% in Caucasians who are at intermediate risk, and 34% in Asian Americans, who are at lower risk. Because of the apparent correlation between the overall racial-ethnic population risk for developing prostate cancer and the frequency for this arbitrarily defined 'short' CAG allele, we[24] postulated that the number of men with short CAG AR alleles should be enriched in a population of prostate cancer patients when compared with a control population. We further hypothesized that since a shorter CAG AR allele encodes for a more transcriptionally active receptor, this could promote tumorogenesis by enhancing prostatic epithelial cell turnover.

In 1995 we directly tested this hypothesis in a small case/control study examining 68 prostate cancer patients and 123 control subjects.[25] As in the study by Edwards et al., there was a high prevalence of short CAG AR alleles among the African-American controls. There was, in addition, a modest, though not significant, enrichment of short CAG AR alleles in the Caucasian prostate cancer population. These supportive results were later confirmed and further extended in an expanded follow-up study utilizing the same prostate cancer patients but a larger control population.[26] A significantly higher prevalence of short CAG AR alleles was noted among prostate cancer patients. This was

especially true for patients with advanced disease, defined as tumors that progressed outside of the prostatic capsule.

Numerous studies have subsequently been published evaluating the role of the AR CAG microsatellite polymorphism and the risk of developing prostate cancer (summarized in Table one). The largest such study, by Giovannucci et al.,[7] used a population selected from the Physicians Health Study. Five hundred and eighty seven prostate cancer cases and 588 controls were selected out of this large cohort of 22,071 U.S., predominantly Caucasian (95%), male physicians, aged 40-84 years old. The large sample size of this study allowed the authors to stratify the case patients by tumor grade and stage. They observed a highly significant inverse correlation between CAG repeat length and the risk of developing prostate cancer when the CAG repeat size was analyzed as a continuous variable. As with our results, short CAG AR alleles correlated with an increased risk of developing advanced disease, defined as a high stage or high grade tumor; however, this correlation did not exist for low stage/grade disease. Thus, a man with an AR CAG repeat length of 19 was 2.5 times more likely to develop high grade/stage prostate cancer than an individual with a CAG repeat size of 25, but no more likely to develop low grade/stage disease.

Stanford et al. in 1997[27] published a different study analyzing CAG repeat length and prostate cancer risk in 301 Caucasian prostate cancer cases and 277 matched controls. This study noted only a small increase in CAG alleles less than 22 repeats in size in the cancer patients when compared with the controls. Nevertheless, when the

CAG repeat size was examined as a continuous variable they observed an overall age-adjusted relative odds of developing prostate cancer of 0.97. In contrast to both our study and that of Giovannucci et al., there was no apparent distinction between the risk of developing localized versus advanced disease among the men in this study.

Another study by Hakimi et al.[28] in Caucasian men, measured the CAG repeat length among 59 prostate cancer patients and 370 controls. Six of the 59 cases (10%) had a short CAG AR allele, that is less than 18 repeats, whereas only 11 of 370 controls (3%) had a short CAG allele (P=0.02). Furthermore, 5 of 6 (83%) patients with short CAG AR alleles had advanced disease, defined as prostate cancer involving the lymph nodes.

More recently, Ekman et al.[29] and Hsing et al.[30] found that CAG alleles were significantly shorter among Swedish and Shanghai Chinese prostate cancer patients compared to controls. Nam et al.[31] found an increased risk associated with shorter CAG repeats only among patients at 'low risk' for recurrence and not for patients at 'high risk' of recurrence. In another recent study Platz et al.[32] used multivariable, pooled logistic regression to adjust for potential dietary and lifestyle factors in a sample of African-Americans, Asians and Caucasians and concluded that the AR CAG repeat difference remained a significant factor in explaining the excess risk of prostate cancer among African-American men. In contrast to the numerous above-mentioned studies, no significant correlations between CAG repeat length and prostate cancer risk were found among French/Germans,[33] British Caucasians,[34] nor among a different cohort of

Swedish men.[35] Also, Lange et al.[36] found no correlation between any prostate cancer parameter and AR CAG repeat length among US Caucasians.

The reasons for the disparate results are not clear, but might be related to the heterogeneous nature of the disease itself or due to unknown covariates or confounders not being taken into account. For example, it is possible that there are other, as yet unidentified, functional genetic changes (like the GGN repeat or single nucleotide polymorphisms) near the CAG microsatellite with which it is in partial linkage disequilibrium. Since such putative variations were not controlled for in the studies referred to above, they could influence the results between studies. Furthermore, prostate cancer patients present with everything from clinically insignificant disease, such as a small focus of Gleason score 2 disease which is biologically indolent and unlikely to impact on a patient's health, to high risk aggressive disease, such as a large Gleason score 10 tumor which has spread beyond the prostate and is likely to be fatal. The relative proportions of high versus low risk disease and localized versus advanced disease most likely vary across studies and could account for at least part of the differences between them. Furthermore, differences may also exist in how the control populations were defined, and how it was determined that the controls did not have prostate cancer. For example, was the absence of prostate cancer only self-reported by the participant, or was PSA and digital rectal examination screening performed, or was the information solely obtained from medical records or tumor registries? The studies also vary with respect to their racial and socioeconomic distribution. For example, many do not contain a significant number of African American subjects while one of the largest is limited to

health professionals. Further methodological differences may include how the short versus long CAG repeat length was defined in different studies, or whether this was treated as a continuous variable. Not all of these studies may have adequately controlled for known or suspected dietary or hormonal risk factors for prostate cancer, such as high fructose intake, high calcium intake, low tomato consumption, cigarette smoking, low physical activity, or high serum androgen levels, all factors known to influence prostate cancer risk. With the number of potential confounding variables, it is perhaps not surprising that these studies have often reached disparate conclusions. A final answer, then, awaits the difficult task of resolving all of these many challenging issues.

While the epidemiologic studies, discussed above, explore the apparent correlation between AR CAG repeat variation and prostate cancer risk, they do not address the molecular mechanisms which underlie changes in AR transactivation activity due to CAG repeat variation. For example, protein partners that interact with the poly-Q stretch are largely unknown at present. Methods addressing such mechanistic studies are currently being developed in many laboratories and should aid in the interpretation of the association results. Such development is necessary to ultimately evaluate the utility of prior knowledge of a man's AR CAG repeat in risk assessments.

Cell culture studies: Several investigators have demonstrated that while AR's with larger CAG repeats have normal ligand binding affinities, they have lower transactivation activity in *in vitro* transient cotransfection studies.[18-23] For example, Chamberlain et al. in 1994[19] showed that an AR construct with 49 CAG repeats had approximately 17%

less transactivation activity on the MMTV promoter than an AR construct with 25 CAG repeats. Similarly, in 1995 Kazemi-Esfarjani et al.[20] observed a 30-40% decrease in transactivation, again on the MMTV promoter, as the CAG repeat size increased from 12 to 40 residues. One study by Gao et al.[37]; however, found blunted AR transactivation activity with either expansion or contraction of the CAG microsatellite from 20 repeats. Although the protein expression levels of AR have been found to be stable across CAG repeat sizes from 9 to 42, at least two studies have found that AR constructs with larger repeats (50-52) are unstable and undergo degradation (possibly in a ligand dependent fashion) producing degradation products of approximately 70-74 kDA.[22, 38]

The moderate poly-Q size effect *in vitro* in AR transactivation activity observed in most studies is thought to be mediated, at least in part, through altered functional interactions with the NR coactivator complex. For example, in transient cotransfection experiments, the relative decrease in AR transactivation activity with increasing CAG size is larger in p160 coactivator mediated enhancement of AR activity than in AR activity measured alone[22]. This effect is most likely secondary to steric hindrance of the interaction of p160 with the AR caused by the increased CAG size. However, the possibility that direct effects mediated via other cofactors, be they cell-, promoter-, and/or AR-specific, cannot be completely excluded. A candidate for such a cofactor is the RAS related G-protein, also known as Ran or AR-associated protein 24, which has been shown to bind to the poly-Q stretch of the AR's N terminal domain and appears to coactivate the AR in a poly-Q size dependent manner.[39] Given this protein's well-described role in the nuclear transport of proteins, it is possible that Ran less efficiently transports AR with a

larger CAG repeat into the nucleus, which may explain the observed poly-Q dependent variation in coactivation.[40] Clearly more studies are needed to clarify this hypothesis.

Materials:

PCR: The materials used for the PCR reactions can be obtained in kit form from any one of many commercial suppliers. The storage and usage conditions are as recommended by the particular supplier. For example, any of many temperature-stable polymerases might be used. The materials for the poly-acrylamide gel electrophoresis are standard and commercially available from many suppliers [note the toxic nature (inhalation and skin exposure) of unpolymerized acrylamide powder and solutions]. The [α-^{32}P]dCTP used in the PCR reactions is radioactive and appropriate exposure protection needs to be taken including Plexiglas shields and the use of safety eyeglasses.

Cell culture transfection materials: Cell culture plastic ware and media are generic and available from many suppliers. The medium is prepared, stored and used as recommended by them. DNA transfection materials and luciferase detection kits are stored and used as recommended by the particular supplier.

Methods:

Methods for the determination of the AR CAG repeat number: Two methods are commonly employed to measure CAG repeat number from genomic DNA of men. The first is manual[25] and the second semi-automated.[7] A point to remember is that the AR is

located on the X chromosome, so that each man has only one allele and thus one size of the CAG repeat. Furthermore, the polymerase amplification of the DNA fragment that contains the CAG repeat results in so-called 'stutter bands' due to slippage of the enzyme during the DNA polymerization. Obviously, size measurements must consider the same band product across samples.

(i) Manual method: The exon 1 CAG repeat of the AR is amplified from about 10 ng of genomic DNA in two rounds of PCR using *Taq* DNA polymerase (Amersham-Pharmacia Biotech, Piscataway, NJ) and nested primers[42]. The outside or first round PCR primers are 5'-GTGCGCGAAGTGATCCAGAA-3' and 5'-TCTGGGACG-CAACCTCTCTC-3'. The inside or second round PCR primers are 5'-AGAGGCC-GCGAGCGCAGCACCTC-3' and 5'-GCTGTGAAGGTTGCTGTTCCTCAT-3'. The first round of PCR consists of 17 cycles of the following steps: 94 C for 1 min; 55 C for 1 min; and 72 C for 1.5 min. The second round, performed with 1 μl of the first round reaction and 2 μCi [α-^{32}P]dCTP, consists of 28 cycles of the following steps: 94 C for 1 min; 66 C for 1 min; and 72 C for 1.5 min. Second round PCR products are then separated on 5% denaturing polyacrylamide gels that are subsequently exposed to X-OMAT film (Eastman Kodak, Rochester, NY). The number of CAG repeats is determined by comparing the size of the predominant PCR product (i.e., the middle radiographic band, Fig. 1) to a series of previously sequenced CAG size standards. All unknown samples from a particular

autoradiogram are then ranked according to relative size and rerun a second time to confirm or modify the original CAG size determination.

(ii) Semi-automated method: The exon 1 CAG repeat of the AR is amplified using fluorescent labeled primers 5'-TCC-AGAATCTGTTCCAGAGCGTGC-3' and 5'-GCTGTGAAGGTTG-CTGTTCCTCAT-3'. Products are resolved and sized utilizing a denaturing polyacrylamide gel and an automated fluorescence detection system (Genescan; Applied Biosystems).

Method for the measurement of AR transactivation activity using transient cotransfection assay[43]: Prostate cancer cells (PC-3, DU145 or LNCaP) are plated into 96-well culture plates at a density of 15,000 cells/well. The next day the cells are transfected with plasmids and Lipofectamine2000 Reagent complexes (Life Technology, BRL). The latter are prepared using two tubes as follows (per well): (i) Mix 25 μl medium with 100 ng reporter gene plasmid (Luciferase under control of an androgen response element containing promoter) and 2.5 ng receptor plasmid (AR expression plasmid) in one tube. (ii) Mix 25 μl medium and 0.4 μl of Lipofectamine2000 in another tube. Tubes (i) and (ii) are combined, mixed and incubated at room temperature 20-30 minutes (transfection solution). Culture medium is removed from the wells, replaced with 50 μl of the transfection solution and incubated for 3-5 hours. The transfection solution is then replaced with 200 μl culture medium and incubated for 36-48 hours. Luciferase activity is then measured in cell lysates using Promega's luciferase reporter kit.

Normally experiments are constructed using quadruplicate wells/experimental point. The total amount of DNA being transfected per well in a particular experiment is balanced across all experimental points by using equivalent molar amounts of all promoter elements (using 'empty' vectors, so that limited general transcription factors are the same across all experimental conditions) as well as equivalent DNA mass [using the promoter-less plasmid pCAT basic (Promega) as 'filler' DNA].

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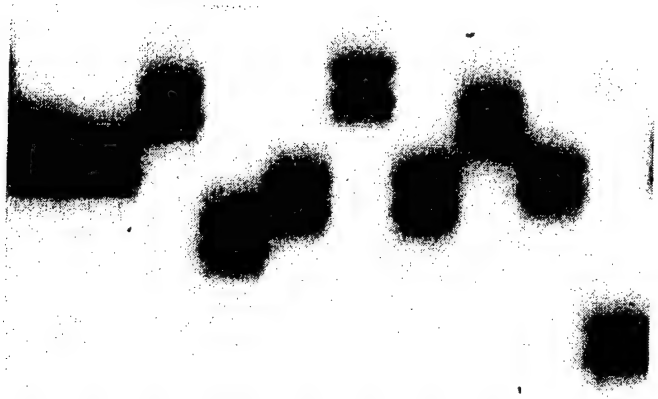
Figure 1: Autoradiogram of CAG amplified products resolved on Urea/PAGE



21 21 24 17 19 25 19 23 20 13

AR CAG repeat size

Table 1: Summary of studies evaluating the role of the AR CAG in prostate cancer risk, progression, and age at onset.

Study	Subjects	risk	stage/grade	age at onset
Irvine et al (95)[25]	US Caucasian	yes (with GGC)	NA	NA
Hardy et al (96)[41]	US Caucasian	NA	no	yes
Ingles et al (97)[26]	US Caucasian	yes	yes	NA
Giovannucci et al (97)[7]	US Caucasian	yes	yes	no
Stanford et al (97)[27]	US Caucasian	yes	no	yes
Hakimi et al (97)[28]	US Caucasian	yes	yes	no
Ekman et al (99)[29]	Swedes	yes	NA	NA
Correa-Cerro et al (99)[33]	French/German	no	no	no
Edwards et al (99)[34]	British	no	no	NA
Bratt et al (99)[35]	Swedes	no	yes	yes
Lange et al (00)[36]	US Caucasian	no	no	no
Hsing et al (00)[30]	Shanghai Chinese	yes	no	NA
Nam et al (00)[31]	Canadian	no	yes	NA

NA = not applicable or not addressed.

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